Analysis of the role of transcription factors in enhancing drought tolerance in sugarcane (*Saccharum* spp.)

by

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Declaration

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TABLE OF CONTENTS

Acknowledgement **VIII**

List of abbreviations **IX**

List of figures **XIV**

List of tables **XXII**

Abstract **XXIV**

Opsomming **XXVI**

PREFACE

Background information XXVIII

Aim and objectives XXX

Chapter layout XXXI

CHAPTER 1:

LITERATURE REVIEW

1.1 Drought and its effect on agriculture **1**

1.2 Sugarcane and drought in Southern Africa

1.2.1 Drought in South Africa 2

1.2.2 Sugarcane in South Africa and its global significance 3

1.2.3 Sustainable sugarcane production in SA under threat due to drought 5

1.3 General physiological effects of water deficit stress on plants

1.3.1 Adjustment of photosynthesis during drought 7

1.3.2 Abscisic acid influence the abiotic stress response in plants 9

1.3.3 Oxidative stress and Reactive oxygen species (ROS) 11

1.3.4 Enzymatic and non-enzymatic antioxidants 12

1.4 Sugarcane improvement

1.4.1 Breeding strategies and its limitations 13

1.4.2 Genetic engineering of sugarcane 14

1.5 Transcription factors (TFs) **16**

1.6 Zinc finger family of proteins **18**

1.6.1 Structure of the zinc finger protein 19
1.6.2 The B-box zinc finger subfamily 20
1.6.3 BBX transcriptional control 21
1.6.4 BBX function roles 22
1.7 NAM, ATAF, CUC (NAC) Transcription factor 23
1.7.1 Structural of the NAC Proteins 24
1.7.2 Function and regulation of the NAC transcription factors 25

CHAPTER 2:

OVEREXPRESSION OF A B-BOX ZINC FINGER TRANSCRIPTION FACTOR IN SUGARCANE TO ENHANCE DROUGHT TOLERANCE

2.1 Introduction 30
2.2 Materials and methods
2.2.1 Chemicals 32
2.2.2 Transgene 33
2.2.3 Standard molecular techniques
2.2.3.1 Plasmid DNA transformation and isolation 33
2.2.3.2 Heat -shock competent cells 33
2.2.3.3 Electro-competent bacterial cells 34
2.2.3.4 Primer design and PCR amplification 34
2.2.3.5 Genomic DNA extraction 35
2.2.3.6 RNA extraction and cDNA synthesis 35
2.2.4 Construction of plant expression vector 35
2.2.5 Plant transformation, selection and regeneration 37
2.2.6 Molecular analysis of putative transformed plantlets
2.2.6.1 Transgene integration 39
2.2.6.2 Transgene expression 39
2.2.7 Analysis of plant phenotype under normal environmental conditions
2.2.7.1 Plant growth 39
2.2.7.2 Carbohydrate content 40
2.2.8 Drought pot trials 40

2.2.9 Physiological and biochemical analysis of plants exposed to drought.

2.2.9.1 Relative water content (RWC) 41

2.2.9.2 Stomatal conductance 41

2.2.9.3 Chlorophyll fluorescence 42

2.2.9.4 Chlorophyll content 42

2.2.9.5 Electrolyte leakage (EC) 42

2.2.9.6 Quantifying malondialdehyde (MDA 43

2.2.9.7 Reactive oxygen species (ROS) 43

2.2.9.8 Catalase (CAT) and superoxide dismutase (SOD) 44

2.2.9.9 Glutathione 45

2.2.9.10 Proline 46

2.2.9.11 Abscisic acid (ABA) 46

2.2.10 Protein subcellular localization

2.2.10.1 Plant expression vector 46

2.2.10.2 Agrobacterium tumefaciens transformation 47

2.2.10.3 Agrobacterium transformation of onion epidermis cells 47

2.3 Results

2.3.1 Transgene cloning into expression vector 49

2.3.2 Confirmation of sugarcane transformation 49

2.3.3 Expression analysis of transgene in transgenic sugarcane 51

2.3.4 Agronomic traits of transgenic and WT plants under non-stressed environmental conditions 52

2.3.5 Phenotypic response of WT and transgenic sugarcane exposed to Drought 55

2.3.6 Lipid peroxidation and membrane damage in transgenic sugarcane under drought stress 60

2.3.7 Oxidative stress in transgenic and WT sugarcane under drought Stress 61

2.3.8 Antioxidant response and compatible osmolyte accumulation in transgenic and WT plants under drought stress 63

2.3.9 Photosynthetic activity in transgenic sugarcane and WT under drought stress 65

2.3.10 ABA accumulation in transgenic and WT plants in response to drought stress 67

2.3.11 Subcellular localization

2.3.11.1 Cloning and transformation of AtBBX29 68

2.2.11.2 Agrobacterium transformation and histology analysis of transient gene expression 70

2.4 Discussion 71

CHAPTER 3:

OVEREXPRESSION OF A TOMATO *NAC2* GENE IN SUGARCANE TO FUNCTIONALLY ANALYSE ITS ROLE IN DROUGHT TOLERANCE

3.1 Introduction 80

3.2 Materials and methods

3.2.1 Transgene 82

3.2.2 Construction of a plant expression vector 82

3.2.3 Plant transformation and tissue culture 83

3.2.4 Confirmation of transgene integration and expression 84

3.2.5 Drought pot trials 85

3.2.6 Protein subcellular localization 86

3.3 Results

3.3.1. Transgene cloning into plant expression vector 89

3.3.2 Confirmation of sugarcane transformation with the *SINAC2* transgene 89

3.3.3 Analysis of transgene expression in transgenic sugarcane 91

3.3.4 Phenotypic response of WT and transgenic sugarcane exposed to Drought 92

3.3.5 Root morphology and biomass of transgenic sugarcane and WT under drought stress 97

3.3.6 Photosynthetic activity in transgenic sugarcane and WT under drought Stress 99

3.3.7 Subcellular localization

3.3.7.1 Cloning and Agrobacterium transformation of SINAC2 100

3.3.7.2 Histology analysis of transient gene expression 102

3.4 Discussion **103**

CONCLUSIONS AND FUTURE PROSPECTS **109**

SUPPLEMENTARY DATA **115**

REFERENCES **123**

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LIST OF ABBREVIATIONS

µF	Microfarad
µg	Microgram
µl	Microlitre
µMol	Micro molar
2.4 D	2,4 Dichlorophenoxyacetic acid
A	Absorbance
ABA	Abscisic acid
ABF	ABRE binding factors
ABRE	ABA-responsive element
APX	Ascorbate peroxidase
ATAF	*Arabidopsis* transcription activation factor
ATP	Adenotriphosphate
BAP	6-Benzylaminopurine
BBX	B-box
bp	Base pair
BSA	Bovine serum albumin
$Ca_2(NO_3)^2$	Calcium nitrate
$CaCl_2$	Calcium chloride
CaMV 35S	Cauliflower mosaic virus 35S
CAT	Catalase
cDNA	Complimentary deoxyribonucleic acid
cm	Centimetre (s)

CO_2	Carbon dioxide
Cu/Zn-SOD	Cupper/Zinc Superoxide dismutase
CUC	Cup-shaped
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DRE	Drought-responsive element
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DW	Dry weight
E.coli	*Escherichia coli*
EC	Electrolyte leakage
EDTA	Ethylenediaminetetraacetic acid
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
g	Grams
g/l	Grams per litre
G45	Geneticin
gDNA	Genomic Deoxyribonucleic acid
GFP	Green fluorescent protein
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSH	Glutathione (oxidized)
H_2O	Water molecule

H_2O_2	Hydrogen peroxide
K_2HPO_4	Potassium hydrogen phosphate
KOH	Potassium hydroxide
kPa	Kilopascals
L	Litre
LB	Lysogeny broth
M	Molar
MDA	Malondialdehyde
MES	2-[N-Morpholino]ethanesulfonic acid
$MgCl_2$	Magnesium chloride
min	Minutes
mM	Milli-molar
Mn-SOD	Manganese superoxide dismutase
MS	Murashige and Skoog
MYB	Myeloblastosis
MYC	Myelocytomatosis
Na_2HPO_4	Disodium phosphate
NAA	1-Napthaleneacetic acid
NAC	NAM, ATAF and CUC
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotine adenine dinucleotide phosphate
NAM	No Apical Meristem

NBT	Nitro blue tetrazolium chlorine
ng	Nanogram
nm	Nanometer
nMol	Nano molar
O_2	Oxygen molecule
O_2^-	Superoxide radical
^oC	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
PVP	Polyvinylpyrolidine
pH	Negative log of hydrogen ion activity
rDNA	Recombinant DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Second (s)
SOD	Superoxide dismutase
SqRT-PCR	Semi-Quantitative Reverse Transcriptase-Polymerase chain reaction
T	Transgenic line
TBA	Thiobarbituric acid
TBE	Tris-Boric acid-EDTA buffer
TCA	Trichloroacetic acid
TF	Transcription factor

TW	Turgid weight
U	Unit (enzyme)
UBi	Maize ubiquitin promoter
UV	Ultraviolet
v/v	Number of ml per 100 ml concentration expression
w/v	Number of grams per 100 ml concentration expression
WT	Wild-type
ww	Without watering
Zn	Zinc

LIST OF FIGURES

CHAPTER 1

Figure 1.1. South Africa annual rainfall between 1970 and 2015, also indicated is the long term average annual rainfall for the period 1970-2015. Source adopted from BFAP (2016), Drought Policy Brief South African Weather Service (Ntombela *et al.,* 2017). 3

Figure 1.2. Major sugarcane producing areas in South Africa including drought disaster provinces. Sugarcane predominantly cultivated in KwaZulu-Natal (KZN) receives water from rainfall while sugarcane in Mpumalanga is irrigated and 14 sugar mills operative through these growing areas (Agri, S.A., 2016). 5

Figure 1.3. A simplified representation where drought conditions limit photosynthesis. Under water-deficit stress abscisic acid is synthesized resulting in stomatal closure and leading to a reduction in intracellular carbon dioxide concentrations which will decrease photosynthesis. Drought also reduce the water content in especially leaf tissue which influence the activity of RuBISCO and other enzymes needed to maintain carbon dioxide fixation and photosynthesis. Image created using https://mindthegraph.com software. 9

Figure 1.4. An illustration of the pathway in which ABA is synthesised from the plastid to the cytosol. ABA synthesis is initiated through the cleavage of β-carotene and the conversion to Xanthoxin which through an intermediate, form ABA-aldehyde (Xiong and Zhu, 2003). 11

Figure 1.5. A three dimensional structure of zinc finger proteins consisting of an alpha helix and beta sheet. The Zn metal ion is coordinated by two histidine and two cysteine residues, typical of a C2H2-type zinc finger protein. The number of residues can vary and result in different fold groups of zinc finger proteins. The images are available at: https://sciencescienceeverywhere.files.wordpress.com/2015/08/figure11.jpg. 21

Figure 1.6. Structure of the NAC protein. The N-terminus of a NAC TF consists of subdomains (A-E) responsible for DNA binding. The C-terminus is the functional domain with transcriptional regulatory or repressor motifs (Ciarmiello *et al.,* 2014). 25

Figure 1.7. Structure of a NAC TF bound to target DNA. The NAC domain is forming a NAC-DNA complex through dimerization with the N-terminus part of another NAC domain (Gonzalez, 2015; Podzimska *et al.,* 2015). 26

CHAPTER 2

Figure 2.1. Colony PCR confirming cloning of the *AtBBX29* gene into the pUbi510 plant expression vector and transformation into the *E. coli* DH5α cells. Lanes M (*Pst*I λ marker); 1-3 (transformed *E. coli* colonies); P (positive plasmid control; pJet1.2:*AtBBX*); NC (negative H_2O control). 50

Figure 2.2. Generating *in vitro* putative transformed sugarcane (*Saccharum* spp hybrid cv. NCo310) plantlets from bombarded embryogenic callus. Callus was initiated from (a) immature inner leave roll explants isolated from stalk material; followed by the (b) development of callus and the harvesting of embryogenic callus (c) prior to transformation. (d) Bombarded callus was placed on selection media, and surviving calli (e) were allowed to develop somatic embryos (f), which developed into sugarcane plantlets (g) that were allowed to elongate and form roots (h). 51

Figure 2.3. PCR analysis confirming the presence of the *AtBBX29* transgene in four of the putative transformed sugarcane lines (T1.1, T1.6, T1.8, and T2.1). M (*Pst*I λ marker); P (positive plasmid control; pJet1.2:*AtBBX29*); NC (negative H_2O control). 52

Figure 2.4. Gel electrophoresis of RNA extracts from the leaves of *AtBBX29* transformed sugarcane (lines T1.1, T1.6 and T1.8,) and WT plants using a Maxwell® 16 LEV Plant RNA Kit. M (*Pst*I λ marker). RNA separation was conducted on a % (w/v) TBE agarose gel, run at 100 V. 53

Figure 2.5. Semi-quantitative real time PCR (SqRT-PCR) showing expression levels of the *AtBBX29* transgene in transgenic sugarcane lines (T1.1, T1.6 and T1.8) and WT plants using **(a)** *Actin* primers as an internal control in comparison with **(b)** gene specific primers. M (*Pst*I λ marker); NC (negative H_2O control). 53

Figure 2.6. Maturing *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants in the growth tunnel on Welgevallen Experimental farm, Stellenbosch. 54

Figure 2.7. Phenotypic growth of *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants measured after 8 months of growth under normal environmental conditions. **(a)** Average leaf length (mm), **(b)** average leaf width (mm), **(c)** average plant height (mm) and **(d)** internodes lengths (mm) were measured. Data is presented as means ± SD of three biological replicates (n = 3). (*) and (**) shows significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test. 55

Figure 2.8. The levels of total soluble sugars in the immature and matured internodes (I 3 and I 9) of *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants measured after 8 months of growth under normal environmental conditions. Measurements include **(a)** sucrose; **(b)** glucose and; **(c)** fructose content. Data is presented as means ± SD of nine biological replicates (n = 9). (*) and (**) shows significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test. 56

Figure 2.9. Phenotypic variation of *AtBBX29* transgenic sugarcane lines (T1.1, T1.6 and T1.8) and wild-type (WT) plants under drought. Drought was initiated by deprivation of water for a period of 21 days**.** Phenotypic analysis was evaluated every 7 days of water deprivation**. (a)** Shows phenotypic changes at days 0, 7, 14 and 21; and **(b)** recovery after re-watering for 14 days. 58

Figure 2.10. Comparative analysis of **(a)** soil moisture content of all pots and **(b)** relative water content (%) in the leaves of transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants prior to and after induction of water-deficit stress. Data is

presented as means ± SD of four biological replicates (n = 4). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determinedusing one-way ANOVA and variance using Tukey's multiple comparison. 61

Figure 2.11. Estimation of cellular damage in transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants under drought stress. Measurement of average **(a)** malondialdehyde (MDA) content and; **(b)** electrolyte leakage. Error bars designates standard deviatio (SD) of the means of three biological repeates (n = 3). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determinedusing one-way ANOVA and variance using Tukey's multiple comparison test. 62

Figure 2.12. Histochemical analysis of reactive oxygen species (ROS) accumulation in the leaves of transgenic (T1.1, T1.6 and T1.8) and WT plants under drought stress, on days 0 to 21 without water. **(a)** Nitro-blue tetrazolium (NBT) staining of superoxide radicals (O_2^-) and; **(b)** 3,3-diaminobenzidine (DAB) staining of hydrogen peroxide (H_2O_2). 63

Figure 2.13. Quantitative analysis of hydrogen peroxide (H_2O_2) levels in transgenic (T1.1, T1.6 and T1.8) and WT plants under drought stress. Data is presented as means ± SD of three biological replicates (n = 3). (*), (**) shows significant difference compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test. 64

Figure 2.14. Estimation of antioxidant and osmolyte activity in transgenic (T1.1, T1.6 and T1.8) and WT plants exposed to water-deficit stress. Measurements include **(a)** superoxide dismutase (SOD) activity, presented as the rate of WST inhibition; **(b)** catalase; **(c)** proline content and; **(d)** glutathione content. Data is presented as means ± SD of three biological replicates (n = 3). (*), (**) and (***) shows significant differencecompared to the WT values at $p \leq 0.05$, 0.01 and 0.001, respectively. Statistical significance: using one-way ANOVA and variance using Tukey's multiple comparison test. 66

Figure 2.15. Analysis of photosynthetic machinery of transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants prior to (day 0) and after exposure to water-deficit stress (days 7, 14 and 21 ww). Measurements include the **(a)** photosynthetic rate (Fv/Fm); **(b)** stomatal conductance and; **(c)** chlorophyll content of the different plants. Data is presented as means ± SD of four biological replicates (n = 4). (*), (**) and (***) indicates significant differences compared to the WT at $p \leq 0.05$, 0.01 and 0.001, respectively. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test. 68

Figure 2.16. Abscisic acid (ABA) content in the leaves of *AtBBX29* transgenic sugarcane and WT plants exposed to water-deficit stress. Data is presented as means ± SD of three biological replicates (n = 3). Significance differences between the genotypes at $p \leq 0.05$ was not observed. 69

Figure 2.17. Diagram illustrating the infusion of the *AtBBX29* gene upstream of the *GFP* reporter gene in the pCambia1302 vector. ZipNCO*I* forw is a primer designed to carry the CCATGG sequence and a ZipNCO*I* rev primer designed to remove the stop codon (**TGA**: 2 bases removed) from the BBX sequence to generate the start codon (**ATG**) of *GFP* in frame with the *GFP* reporter gene. 70

Figure 2.18. Colony PCR analysis confirming the cloning of the **(a)** full length *AtBBX29* gene (lanes 1-6) into the pCambia1302 plant expression vector and transformation into the *E. coli* DH5α cells; and **(b)** transformation of pCambia1302:*GFP*:*AtBBX29* into *Agrobacterium tumefaciens* (lanes 1-3). Lane M (*Pst*I λ marker); P (positive plasmid control; pJet1.2: *AtBBX29*); NC (negative H_2O control). 70

Figure 2.19. Histology analysis of the transient gene expression of GFP to determine the subcellular localization of the *AtBBX29* gene in onion epidermis cells transformed with the **(a)** pCambia1302:*GFP* control vector and; **(b)** pCambia1302:*GFP:AtBBX29* recombinant vector construct. 71

CHAPTER 3

Figure 3.1. Colony PCR confirming cloning of the *SINAC2* gene into the pUbi510 plant expression vector and transformation into the *E. coli* DH5α cells. Lanes M (*Pst*I λ marker); 1-9 (transformed *E. coli* colonies); P (positive plasmid control; pJet1.2:*SINAC2*); NC (negative H_2O control). 90

Figure 3.2. Generating *in vitro* putative transformed sugarcane (*Saccharum* spp hybrid cv. NCo310) plantlets from bombarded embryogenic callus. Callus was initiated from (A) immature inner leave roll explants isolated from stalk material; followed by the (B) development of callus and the harvesting of embryogenic callus (C) prior to transformation. (D) Bombarded callus was placed on selection media, and surviving calli (E) were allowed to develop somatic embryos (F), which developed into sugarcane plantlets (G) that were allowed to elongate and form roots (H). 91

Figure 3.3. PCR analysis confirming the presence of the *SINAC2* transgene in five of the putative transformed sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5). M (*Pst*I λ marker); P (positive plasmid control; pJet1.2:*SINAC2*); NC (negative H_2O control). 92

Figure 3.4. Gel electrophoresis of RNA extracts from the leaves of the leaves of *SINAC2* transformed sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5) and WT plant using a Maxwell®16 LEV Plant RNA Kit. M (*Pst*I λ marker); (NC) (negative H_2O control). RNA separation was conducted on a 1% (w/v) TBE agarose gel, run at 100 V. 93

Figure 3.5. Semi-quantitative Real Time PCR (SqRT-PCR) showing expression levels of *SINAC2* transgene in transgenic sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5) and WT plant using **(a)** *Actin* primers as an internal control in comparison with **(b)** gene specific primers. M (*Pst*I λ marker); NC (negative H_2O control). 93

Figure 3.6. Phenotypic responses of *SINAC2* transgenic sugarcane lines (T1.1, T1.2 and T1.5) and WT plants to extended drought condition. Drought was initiated by

deprivation of water to all plants for a period of 31 days**. (a)** Shows phenotypic changes of transgenic lines and WT plants at day 0, 14, 21, 28 and 31 without water (ww). **(b)** Phenotypic recovery after re-watering for 14 days. 95

Figure 3.7. Comparative analysis of **(a)** soil moisture content of all pots and **(b)** relative water content (%) in the leaves of transgenic sugarcane lines (T1.1, T1.2 and T1.5) and wild-type (WT**)** plants prior to and after induction of water deficit stress. Data is presented as means ± SD of three biological replicates (n = 3). (**) and (***) shows significant differences compared to the WT at $p \leq 0.001$ and $p \leq 0.0001$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison Test. 98

Figure 3.8. Comparison of the root growth of *SINAC2* transgenic sugarcane (T1.1, T1.2 and T1.5) and WT plants under 28 and 31 days without water. 99

Figure 3.9. Analysis of photosynthetic machinery of transgenic sugarcane (T1.1, T1.2 and T1.5) and wild-type (WT) plants prior to (day 0) and after exposure to water-deficit stress. Measurements of **(a)** photosynthetic rate (Fv/Fm) and; **(b)** Stomatal conductance. Data is presented as means ± SD of four biological replicates (n = 6). (*) and (**) shows significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.001$, respectively. Statistical significance was determined using one-way ANOVA and varience using Bonferroni's multiple comparison test. 101

Figure 3.10. PCR analysis confirming the full length *SINAC2* amplicon entry into the pENTRY vector (lanes 1-4; amplicon size 964 bp) into OneShot®Top10 *E. coli* cells. M (*Pst*I λ marker); and NC (negative H_2O control). 102

Figure 3.11. PCR analysis confirming cloning and transformation of pENTRY:*SINAC2* into OneShot®Top10 *E. coli* cells, where clones were tested with M13 vector specific (A; amplicon size 1305 bp); ENTRY cloning (B; amplicon size 946 bp); and two clones with *SlNAC* specific (C; amplicon size 600 bp) primers sets. M (*Pst*I λ marker); and NC (negative H_2O control). 102

Figure 3.12. Colony PCR analysis confirming sub-cloning of *SINAC2* into a pGWB506 destination vector and transformation into OmniMAX *E. coli* cells. M (*Pst*I λ marker); NC (negative H_2O control); P (positive plasmid control; pJet1.2:*SINAC2*). 103

Figure 3.13. Histology analysis of the transient gene expression of *GFP* to determine the subcellular localization of the *SINAC2* gene in onion epidermis cells transformed with the pGWB506:*SINAC2* recombinant vector construct. Images were visualized on an A1 microscope connected to ZEISS Axiocam 305 color imaging software at 10x magnification. 104

LIST OF TABLES

CHAPTER 1

Table 1.1 NAC transcription factors which were identified in a variety of crops and have been associated with regulation of stress responsive genes to enhance tolerance to drought. 29

CHAPTER 2

Table 2.1 PCR primers used for the cloning and screening of the *AtBBX29* transgene. 38

Table 2.2 Leaf width, length and shoot height of *AtBBX29* transgenic and WT plants evaluated every 7 days for the duration of the 21 day water-deficit period. Values present the average means ± standard deviation (SD) of four biological replicates (n = 4). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test. 59

Table 2.3 Survival rates (%) of *AtBBX29* transgenic sugarcane and WT plants exposed to 21 days of drought. For each genotype a total of 25 plants were included in the pot trial. Plants were recorded as healthy when less than 5% of the leaves showed signs of browning. Values represent the number of plants being dead, damaged or healthy at each time point, expressed as a percentage. 60

CHAPTER 3

Table 3.1. PCR primers used for *SlNAC2* and *Actin* gene isolation and detection. 86

Table 3.2. PCR primers used for *SlNAC2* cloning into the pGWB506 destination vector. 89

Table 3.3. Leaf width, length and shoot height of *SINAC2* transgenic sugarcane lines and wild-type (WT) plants evaluated every 7 days for the 21 days without water (ww). Values present the average means ± standard deviation (SD) of six biological replicates (n = 6). (*) and (***) shows significances between means of the WT at $p \leq 0.05$ and $p \leq 0.0001$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test. 96

Table 3.4. Survival rates of *SINAC2* transgenic sugarcane (lines T1.1, T1.2 and T1.5) and WT plants exposed to 35 days of drought. For each genotype a total of 25 plants were included in the pot trial. The plants were assessed for damage after 25 days without water (ww) and again every second or third day after this time point. Plants were recorded as healthy when less than 5% of the leaves showed signs of browning. Values represent the number of plants being dead, damaged or healthy at each time point, expressed as a percentage. 97

Table 3.5. Root biomass of *SINAC2* transgenic sugarcane line and wild-type plants measured during the 31 days of water deprivation. Values present the average means ± standard deviation (SD) of three biological replicates (n=3). Statistical variance was determined using Bonferroni's multiple comparison test. 100

ABSTRACT

Sugarcane is a large perennial grass of the genus *Saccharum.* Economically, this grass species is an important source of sugar for food purposes and biomass for biofuel production. However, the sustainability of production is greatly constrained by drought, which directly affects crop yield. Drought can lead to modification of metabolic processes in the plant, membrane disorder, disruptions and instabilities of many physiological and biochemical process, including photosynthesis and increased production of reactive oxygen species (ROS), which causes oxidative stress and can ultimately lead to plant death. It is therefore essential to continue developing cultivars with improved drought tolerance, which can possibly be achieved through the identification and introduction of genes that confer tolerance in crops.

Transcription factors (TF) are gene regulators that control gene expression and consequently stress responses in plants. A single TF can regulate the expression of many target genes. This study aims at analysing the role of two TFs namely, BBX (B-Box Zinc Finger) and NAC2 (NAM, ATAF and CUC) in enhancing drought tolerance in sugarcane. For this, sugarcane was independently genetically transformed via particle bombardment with a BBX TF from *Arabidopsis thaliana* and a NAC2 TF from tomato. Attempts were also made to determine the subcellular localization of the *AtBBX29* and *SINAC2* genes but results were inconclusive due to poor microscopic imaging and faint *GFP* reporter signals. Transgene insertion was confirmed in putative transformed sugarcane through PCR analysis and transgene expression through semi-quantitative reverse transcriptase PCR. Transgenic sugarcane plantlets were planted *ex vitro* and drought pot trials were setup in the glasshouse. Once plants were deprived of water, phenotypic changes in transgenic sugarcane lines were compared to non-transgenic control sugarcane plants.

Under drought conditions, both *AtBBX29* and *SINAC2* overexpression in sugarcane enhanced drought tolerance. All transgenic plants exhibited higher survival and recovery rates than wild-type (WT) plants. Transgenic plants overexpressing *AtBBX29* maintained relative water content (RWC) at levels not significant different from the WT plants. However, these plants maintained significantly higher chlorophyll fluorescent rates and stomatal conductance

under mild and severe drought conditions. Under severe water-deficit stress, oxidative damage was reduced in BBX transgenic plants which exhibited low malondialdehyde (MDA) levels and less accumulation of reactive oxygen species (ROS) throughout the water-deficit stress period. The scavenging activity of antioxidants, which was present at significantly higher levels in the transgenic plants under severe water-deficit stress, most likely played a role in reducing the ROS levels. Transgenic plants also accumulated significantly more proline under mild and severe stress conditions compared to the WT plants. Abscisic acid levels varied between WT and transgenic plants exposed to drought. In addition, BBX transgenic sugarcane was grown to maturity (8 months) under a normal watering regime in the glasshouse where these plants displayed normal phenotypes and no significant difference in carbohydrate content compared to non-transgenic control plants.

A preliminary drought trial was conducted with the *SINAC2* transgenic sugarcane plants. Overexpression of *SINAC2* enhanced drought tolerance in transgenic sugarcane plants exposed to water-deficit stress with higher survival rates seen in the transgenic lines compared to the WT plants. Transgenic plants overexpressing *SINAC2* maintained significantly higher RWC levels and displayed less visual damaged such as leaf wilting and yellowing than the WT plants. Over the course of water-deficit period the root biomass increased in all genotypes, but less so in the transgenic plants, *SINAC2* overexpression however enhanced root elongation. Transgenic plants also upheld photosynthesis, with high chlorophyll fluorescence and stomatal conductance seen in most transgenic plants under severe water-deficit stress.

OPSOMMING

Suikerriet is 'n groot meerjarige gras spesie van die genus *Saccharum*. Ekonomies is hierdie grassoort 'n belangrike suikerbron vir voedseldoeleindes en biomassa vir biobrandstof produksie. Die volhoubaarheid van produksie word egter baie beperk deur droogte, wat die oesopbrengs direk beïnvloed. Droogte kan lei tot verandering in metaboliese prosesse in die plant, membraan-ontwrigting, inhibering en versteurings van baie fisiologiese en biochemiese prosesse, insluitend fotosintese en verhoogde produksie van reaktiewe suurstofspesies (ROS), wat oksidatiewe skade veroorsaak en uiteindelik tot die dood van die plante kan lei. Dit is dus belangrik om voort te gaan met die ontwikkeling van kultivars met 'n verbeterde droogtetoleransie, wat moontlik bereik kan word deur die identifisering en invoeging van gene wat verdraagsaamheid verleen in gewasse.

Transkripsiefaktore (TF) is geenreguleerders wat geenuitdrukking beheer en gevolglik stresreaksies by plante. 'n Enkele TF kan die uitdrukking van baie teikengene reguleer. Hierdie studie het ten doel om die rol van twee TF's, naamlik BBX (B-Boks Sink Vinger) en NAC2 (NAM, ATAF en CUC) te analiseer in die verbetering van die droogtetoleransie in suikerriet. Hiervoor is suikerriet onafhanklik geneties getransformeer via deeltjiebombardering met 'n BBX TF van *Arabidopsis thaliana* en 'n NAC2 TF van tamatie. Pogings is ook aangewend om die sub-sellulêre lokalisering van die *AtBBX29*- en *SINAC2*-gene te bepaal, maar die resultate was onoortuigend as gevolg van swak mikroskopiese beelde en flou *GFP*-verslagseine. Transgeeninplanting is bevestig in vermeende getransformeerde suikerriet deur middel van PKR-analise en transgeenuitdrukking deur middel van semi-kwalitatiewe tru-transkriptase PKR. Transgeniese suikerrietplantjies is *ex vitro* geplant en droogtepotproewe is in die glashuis opgestel. Nadat plante van water ontneem is, is fenotipiese veranderinge in transgeniese suikerrietlyne vergelyk met nie-transgeniese kontrole suikerrietplante.

Onder droogtetoestande het die ooruitdrukking van beide *AtBBX29* en *SINAC2* in suikerriet droogtetoleransie verhoog. Al die transgeniese plante het hoër oorlewings- en herstelsyfers getoon as die wildtipe (WT) plante. Transgeniese plante wat *AtBBX29* ooruitdruk, het die relatiewe waterinhoud (RWC) gehandhaaf op vlakke wat nie noemenswaardig verskil van die

WT-plante nie. Hierdie plante het egter 'n beduidende hoër chlorofil fluoresensie koers en stomale-geleiding getoon onder droogtestres toestande. Onder hoë watertekort spanning, is oksidatiewe skade verminder in BBX transgeniese plante wat lae vlakke van malondialdehied (MDA) getoon het en minder opeenhoping van reaktiewe suurstofspesies (ROS) gedurende die strestydperk. Die de-aktiveringsaktiwiteit van antioksidante, wat teenwoordig was op aansienlike hoër vlakke in die transgeniese plante onder ernstige watertekort, het waarskynlik 'n rol gespeel in die verlaging van die ROS-vlakke. Transgeniese plante het ook aansienlik meer prolien opgebou onder matige en hoë strestoestande in vergelyking met die WT-plante. Absisieksuur vlakke het baie gewissel tussen WT en transgeniese plante wat aan droogte blootgestel was. Boonop is BBX-transgeniese suikerriet opgegroei tot volwassenheid (8 maande) onder normale besproeiing in die kweekhuis, waar die transgeniese plante normale fenotipes vertoon het en daar was geen noemenswaardige verskil in koolhidraatinhoud in vergelyking met nie-transgeniese kontrole plante nie.

'n Voorlopige droogtestudie is met die *SINAC2*-transgeniese suikerrietplante gedoen. Ooruitdrukking van *SINAC2* het die droogtetoleransie verhoog by transgeniese suikerrietplante wat blootgestel was aan watertekorte, met hoër oorlewingsyfers in vergelyking met die WT-plante. Transgeniese plante het aansienlik hoër RWC-vlakke gehandhaaf en minder visueel skade getoon, soos blaarverwelking en vergeling, as die WT-plante. Gedurende die periode van watertekort het die wortelbiomassa by alle genotipes toegeneem, maar minder in die transgeniese plante, *SINAC2* ooruitdrukking het egter die wortelverlenging verhoog. Transgeniese plante het ook fotosintese gehandhaaf, met 'n hoë chlorofil fluoresensie en stomale-geleiding wat by die meeste transgeniese plante onder ernstige watertekorte gesien is.

PREFACE

BACKGROUND INFORMATION

Plants, as one of the fundamental essential food resources have great global commercial significance (Akinci and Lösel, 2012). Plants are sessile organisms exposed to a variety of environmental stimuli and whose life is directed by an array of physiological, biochemical and molecular processes. Crop and other plants are regularly subjected to abiotic stresses, which affect food and forage crop productivity. These stresses cause major alteration in several physiological, cellular and molecular functions of the plant and require adaptation to withstand these effects in comparison with crops grown under non-stressed conditions (Mittler, 2006). Abiotic stress includes numerous stresses caused by complex environmental conditions, such as salinity, deficiency/excess of minerals, drought and temperature extremes (Hirayama and Shinozaki, 2010). These stresses are an impediment for plants as they prevent them from realising their maximum genetic potential by hindering their normal metabolism. Abiotic stresses are the foremost cause of crop loss for crop species in the cereal and grass *Poaceae* family (Tester and Bacic, 2005).

One of the leading abiotic factors that severely impacts agricultural economies and food security is drought, which will most likely increasingly effect crop production in the future due to climate change (Dube and Jury, 2003; Lesk *et al.*, 2016). In many parts of the world, South Africa especially, drought is a serious agronomic problem which can greatly influence the productivity of many crops and affect growth and final yields (Boyer, 1982; Dube and Jury, 2003). Drought affects crop production by decreasing the growth rate, stem elongation, leaf development and stomatal control (Anjum *et al.,* 2011). Droughts and multiple dry spells are common in South Africa. For example, in the 2015/2016 and 1992 production season, southern Africa experience severe droughts (Dube and Jury, 2003; Nhamo *et al.,* 2019). Furthermore, in the past few decades in South Africa, drought had led to the increased need for import of crops, a sharp increase in food prices and the recovery from these takes years (Stevens and van Koppen, 2015; Woods *et al.,* 2008).

Sugarcane (*Saccharum* spp. hybrids), a valuable tropical or sub-tropical grass crop that is mostly cultivated for sucrose production is also severely influenced by water-deficit caused by frequent and severe droughts (Robertson *et al.*, 1999; Silva *et al.*, 2008). Water-deficit stress can reduce sugarcane photosynthetic rates significantly resulting in biomass reductions of up to 50% and can also negatively influence sugar quality (Ferreira *et al.,* 2017; Khaled *et al.,* 2018).

In the past few decades, a great deal of effort has been devoted to breeding crops with various stress-tolerant traits. Traditional approaches to breeding crop plants with improved abiotic stress tolerance tends to be limited due to a focus on yield, sugar content in the case of sugarcane specifically, specific traits oversight, the complexities of genomes and the limitation of crossing only closely related species (Richards, 1996). In addition, most cereals and grasses have limited genetic variability in their gene pool, which may offer few occasions for major step changes in tolerance via traditional breeding attempts (Tester and Bacic, 2005). To supplement conventional breeding, plant biotechnology techniques such as genetic engineering have the ability to target, select and alter key genes to produce novel phenotypes. To date there have been numerous reviews published summarising the options for inducing abiotic stress tolerance, nevertheless the actual production of transgenic plants with palpable enhanced abiotic stress tolerance has been slow. This delay is in part due to the frequently observed negative impact of transgenes on plant growth and/or yield under non-stress environments, as well as the need to manipulate cascades of genes potentially involved in improving tolerance during transgenic alterations (Tester and Bacic, 2005).

Transcription factors (TF) are proteins that influence the changes in gene expression through a unique recognition of *cis*-acting elements that are located on the promoter region of target genes (Century *et al.,* 2008; Nakashima *et al.,* 2009). Ultimately, such regulatory genes are essential for many facets of plant growth and development e.g. cell differentiation, development of tissue and organs, responses to hormones and environmental factors, metabolic networks and disease resistance (Gonzalez, 2015). Hence they are potential targets for gene manipulation to enhance drought tolerance in crop species. Some known TFs namely, APETALA2/ethylene-responsive element-binding protein

(AP2/EREBP), myeloblastosis (MYB), myelocytomatosis (MYC), WRKY (pronounced “worky”), NAM, ATAF and CUC (NAC), zinc finger and basic leucine zipper (ZIP) proteins (Wang *et al.,* 2016a), have been identified and analysed in model species. Transcriptome analysis confirms that an up-regulation of these TFs have an influential role towards plant tolerance to stresses (Gahlaut *et al.,* 2016).

PROJECT AIM AND OBJECTIVES

In this project, a targeted approach directed at the genetic manipulation of transcription factors (TF), linked to abiotic stress responses in plants, will be investigated. The overall aim is to increase drought tolerance in sugarcane by independently overexpressing two TFs which altered gene expression and ultimately the stress response in the plant. The targeted transgenes include a NAC2 TF (NAM, ATAF1, 2 CUC2 multifunctional plant TF family), isolated from tomato and a BBX (B-box zinc finger) TF isolated from *Arabidopsis thaliana.* The following objectives were included in the project:

1. Clone transgenes, *SlNAC2* and *AtBBX29*, independently into the pUbi510 plant expression vector.
2. Genetically transform sugarcane with the pUbi510:*SlNAC2* and pUbi510:*AtBBX29* vectors using particle bombardment.
3. Molecular analysis of putative transgenic sugarcane by confirming transgene insertion and expression.
4. Assessment of transgenic plants for drought tolerance, focusing on physiological and biochemical parameters.
5. Agronomic traits assessment of transgenic plants under non-stress environmental conditions
6. Determine sub-cellular localization of transgenes.

CHAPTER LAYOUT

This MSc thesis consists of three chapters:

Chapter 1 reviews existing literature on the importance of the South African sugar subsector and agricultural activities of sugarcane and their significant contribution to the nation's economy. It emphasise the frequent occurrence of long-term droughts caused by El Nino Southern Oscillation (ENSO) in Southern Africa and the long-term effects it has on agricultural produce, livelihoods, economies and the environment. It also looks at the genetic improvement of sugarcane through traditional breeding and its significant limitations. The use of genetic engineering, which can improve both yield and tolerance to adverse environmental factors and how it has been successfully implemented on a number of crops comprising complex genomes like the *Saccharum spp*. This chapter reviews recent progress on the manipulation of gene(s), especially transcription factors (TFs) (BBX and NAC), which regulates the expression of a multitude of genes able to stimulate a series of physiology and morphology changes in plants and their associated role in enhance drought tolerance in plants.

Chapter 2 is composed of an introduction on the BBX transcription factor and its role in transgenic crops to significantly enhance drought tolerance. It covers the experimental work of generating transgenic sugarcane plants through, first cloning of the *AtBBX29* gene into the pUbi510 plant expression vector, where the transgene is under the control of a ubiquitin promoter, and subsequently transformation into sugarcane callus through particle bombardment. This is followed by the *in vitro* regeneration of putative transgenic sugarcane plantlets and molecular confirmation of transgene integration and expression in the plantlets through PCR and semi-quantitative reverse transcriptase PCR analysis. The chapter then focuses on the physiological and biochemical changes occurring in transgenic lines in comparison to the wild-type plants, when exposed to water-deficit stress. Additionally, an attempt was made to establish the subcellular localization of the *AtBBX29* gene, using a *GFP* reporter gene present in the pCAMBIA1302 binary vector, which was transformed into

Agrobacterium tumefaciens and used to further infiltrate onion epidermis cells for histological analysis.

A focused version of this chapter ("Overexpression of *AtBBX29* improves drought tolerance by maintaining photosynthesis and enhancing the antioxidant and osmolyte capacity of sugarcane plants") has been submitted for publication in the Plant Molecular Biology Reporter journal (Submission ID: PMBR-D-20-00146). We are currently waiting for reviewer feedback.

Chapter 3 focuses on the NAC2 transcription factor, with the chapter comprising of an introduction on the functional analysis of NAC2 TFs in enhancing drought tolerance in crops. The materials and methods summarizes the generation of *SlNAC2* transgenic sugarcane plant lines through bombardment and molecular analysis of *SlNAC2* transgene integration and expression in the genome of transgenic sugarcane though PCR and sqRT-PCR analysis. Data is presented on a preliminary glasshouse drought pot trial, which includes the physiological analysis of the *SlNAC2* transgenic sugarcane lines. Analysis included the evaluation of the physiological mechanisms such as chlorophyll fluorescence and stomatal conductance in these plants. Additionally, this chapter attempts to determine the subcellular localization of the *SlNAC2* gene by making use of onion epidermis cell transformed with an enhanced *GFP* (e*GFP*) reporter gene, cloned into the pGWB506 binary vector.

Lastly, the **Conclusion and Future Prospects** section summarizes and draw conclusions of the findings of this study. It also suggests future experiments using transgenic sugarcane plants modified with *AtBBX29* and *SlNAC2*. It is particularly important to complete any biochemical and genetic analysis of the *SlNAC2* transgenic plants that can still contribute to the functional analysis of this transgene.

CHAPTER 1: LITERATURE REVIEW

1.1 DROUGHT AND ITS EFFECT ON AGRICULTURE

Agriculture is a major consumer of water resources on the planet as its many operations relies on the probability of rainfall received or water supplied (Ntombela *et al*., 2017). With increasing aridness and a growing human population, predicted to increase with a further 2.3 billion by the year 2050, water will however become an even scarcer commodity in the near future (Hussain *et al.*, 2008). In addition, according to Edossa *et al*., (2014) drought is expected to increase particularly in regions already prone to droughts and this translates into more frequent and severe droughts in especially Southern Africa.

Drought can be described as a period of unusually low rainfall, leading to shortage and imbalances in water availability. The lack of precipitation, reduced soil moisture and decreasing ground water and river flow during a drought has a detrimental effect on crop production. Plants experience water deficit stress when water loss through transpiration exceeds the ability of roots to absorb water and internal water content decreases to levels that will interfere with normal plant processes. This has serious consequences for sustainable agricultural production, which affects the distribution of crop plants and often leads to reduce plant development and yield losses (Hasanuzzaman *et al*., 2013; Nellemann, 2009; Vorosmarty *et al*., 2000). Water scarcity can be a consequence of natural causes e.g. drought due to temporal water shortage, or induced by human activities e.g. aridity due to permanent desertification (e.g. damaged soil, overexploitation of groundwater and degraded water quality due to incorrect use of land), and/or may results from both interactions (Paulo and Pereira, 2006). While drought effects are often measured in terms of crop losses, related issues such as decreased levels of employment, income, nutrition and health are also affected (Ntombela *et al*., 2017; Vogel, 1994).

Water-deficit stress modifies crop physiology by altering the physical environment in which the plant grow, where the plant loses water or the availability of soil moisture is decreased by evapotranspiration due to atmospheric conditions (Akinci and Lösel, 2006; Jaleel *et al.*, 2009). Although it is not possible to avoid drought, its impacts can be handled to an extent through preparations, planning and management (Edossa *et al.,* 2014). It is therefore

imperative to enhance our understanding of the effect of drought on plants to improve systems management and breeding efforts in the agricultural section and for envisaging the influence of climate change on natural vegetation, and to ensure high crop yields under drought (Hussain *et al.,* 2008).

1.2 SUGARCANE AND DROUGHT IN SOUTH AFRICA

1.2.1 Drought in South Africa

The Republic of South Africa is a semi-arid tropical country with climate extremes imposed by long-term drastic climate changes and is prone to frequent drought (Leichenko and O'brien, 2002; Usman and Reason, 2004). The occurrence, length and intensity of drought in South Africa are often unpredictable and South Africa is extremely exposed to the effects of drought. Drought has severe ecological and economic consequences (Vetter, 2009). Historically, according to the South African Weather Service (SAWS), SA received an average annual rainfall of 608 mm. However, during the most recent drought, which occurred in 2015, SA only received 403 mm rain, which is 66% of the annual average rainfall and was the driest year since 1904 (Figure 1.1). The worst affected provinces included the Northern Cape, Limpopo, Free State, KwaZulu-Natal and North West (Agri SA, 2016). This drought had serious consequences including agricultural losses with recorded crop failure at 50% and reduced dam levels which dropped from an average of 70% in 2015 to less than 40% in 2016 (Agri SA, 2016; Vogel, 1994). Consequently, South Africa experienced the worst drought in the 2015/2016 production year caused by an El Nino episode that occurred since middle 2015 (Figure 1.1) (Ntombela *et al*., 2017).

El Nino generally causes severe drought conditions to Southern Africa, Africa in general and countries such as Australia, South-Eastern Asia, China and South America. On record, in Southern Africa, El Nino driven droughts were reported in the 1956 to 1958, 1982 and again in the 1991/1992 seasons (Harsch, 1992). These droughts resulted in reduced livestock production, due to depletion of natural grazing fields, up to 80% decrease in ground biomass production of major grass species such as maize, wheat, soybean and sugarcane

(Tester and Bacic, 2015), reduced sales in the agricultural sector, increased inflation, and negatively affected employment (Agri SA, 2016)

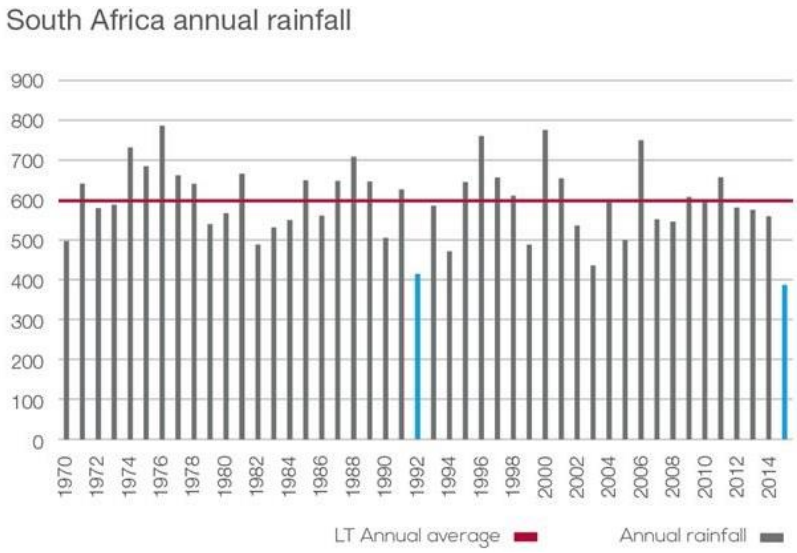

Figure 1.1. South Africa annual rainfall between 1970 and 2015, also indicated is the long-term average annual rainfall for the period 1970-2015. Blue bars indicate the lowest annual rainfall within the reported period. Source adopted from BFAP (2016), Drought Policy Brief South African Weather Service (Ntombela *et al.,* 2017).

1.2.2 Sugarcane in South Africa and its global significance

Sugarcane is a large perennial true grass of the genus *Saccharum.* Species of *Saccharum* are genetically complex and are categorized by a high degree of polyploidy and common aneuploidy in addition to a large genome size (1 Gb; 2n = 115) (Cordeiro *et al.*, 2000; De Setta *et al.*, 2014). This grass belongs to the *Poaceae* family and together with sorghum and maize comprise the Panicoidae super-family with a C4 photosynthetic metabolism (De Setta *et al.*, 2014; Grivet and Arruda, 2002).

All of the existing sugarcane *(Saccharum spp*. hybrid) cultivars at present are the products of ongoing cross breeding since the late 19th century. The species contributing to the varieties were *S. officinarum* (2n = 80)*,* a species known to produce abundant sugar and *S. spontaneum* (2n = 40 - 128), which contributed genes for resistance to several pests and abiotic stresses (Cordeiro *et al*., 2000; Grivet and Arruda, 2002), resulting in cultivars with higher yields and disease resistance (Lakshmanan *et al.,* 2005).

Globally, sugarcane is a unique agricultural crop, the world's largest crop by production quantity, important for food and as a source of biomass for bioelectricity and second-generation bioethanol production (Grivet and Arruda, 2002; Chen *et al*., 2017; Lakshmanan *et al.,* 2005). The primary use for sugarcane is to produce sugar, which accumulates as sucrose in the stalk internodes (Sachdeva *et al.,* 2011; www.daff.gov.za). Sugarcane is commercially grown in tropic and subtropical regions all over the world, in more than 100 countries characterised by warm temperatures, moist climates and deep fertile soil (Mattiello *et al.,* 2015). The largest producers are Brazil (da Silva *et al.,* 2013), India and China (Grivet and Arruda, 2002; Scortecci *et al*., 2012; Waclawovsky *et al.,* 2010; Zhao and Li, 2015).

In South African, the sugar industry is one of the leading cost competitive producers of sugar, contributing 11% to agri-employment while creating around 79 000 jobs (Agri SA, 2016). According to the South African Sugarcane Association (SASA), its contribution to the national economy can be defined based on its annual production of approximately 20 million tons of sugarcane resulting in an average of R8 billion direct income, R3 billion from export earnings per annum, and an estimated R5 billion to the country's foreign exchange incomes. In addition, the 35 500 registered sugarcane growers comprising of about 83% commercial scale farmers, 10% small scale farmers and the remaining is covered by sugar mills firms. South Africa is therefore, the leading producer of sugar in Africa and ranked in the top 15th of the top sugarcane producers' worldwide (www.daff.gov.za). Economic interest in sugarcane has increase significantly in recent years due to increased worldwide demand for sugar and for production of renewable energy (Cheavegatti-Gianotto *et al.*, 2011).

The sustainability of this crop's global production is critically dependent on improving tolerance to various types of environmental stresses. Sugarcane growth and development are highly sensitive to water-deficit stress (Lakshmanan and Robinson, 2014). It has a high water use efficiency for optimal growth and drought is one of the important factors which lead to sugarcane losses in productivity worldwide (Silva *et al.,* 2007). The annual water requirement for sugarcane crop production ranges from approximately 1500 to 2500 mm (Inman-Bamber, 2004; Robertson and Muchow, 1994; Robertson *et al.,* 1997).

1.2.3 Sustainable sugarcane production in SA under threat due to drought

In South Africa only 20% of the arable land area suitable for sugarcane production is irrigated, while the remaining 80% is rain-fed. These regions include the Mpumalanga and the Northern Pondoland in the Eastern Cape (Figure 1.2). In addition, the 12-month crop growth cycle do not allow producers to avoid the annual dry season.

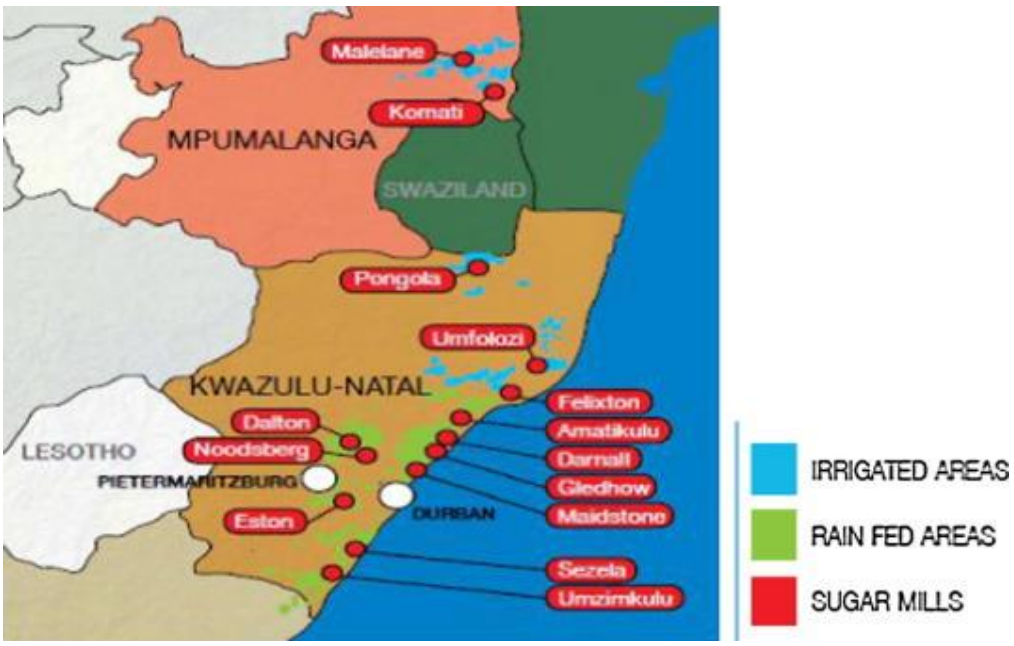

Figure 1.2. Major sugarcane producing areas in South Africa including drought disaster provinces. Sugarcane is predominantly cultivated in KwaZulu-Natal (KZN) which receives water from rainfall while sugarcane in Mpumalanga is irrigated and 14 sugar mills operative through these growing areas (Agri, S.A., 2016).

In South Africa, KwaZulu Natal (KZN) beside the east coast is classified as a summer rainfall region (Dube and Jury, 2003). However, it is among the drought prone regions in SA and have suffered a double season of drought in the year 2010/2011 and 2014/2015, which resulted in a substantial decline in cane production. For example, in the 2015 drought, the KZN sugarcane production was reduced by 34% when compared with the 2013/2014 production season (www.daff.gov.za).

Drought stress can affect sugarcane growth in several ways depending on the severity and duration of the stress, and the level of plant growth and genotype when the stress is applied.

Sugarcane development is categorized in stages that includes germination, tillering, grand growth and maturity (Ferreira *et al.,* 2017). Each of these stages is affected differently by water-deficit. The germination phase start 7-10 days of planting and result in the sugarcane stalk sprouting buds and initiating roots, and usually lasts up to ± 35 days (Zingaretti *et al.,* 2012). Tillering is a physiological process of underground repeated branching of shoots which ensures good yield by providing a suitable quantity of stalks necessary to build adequate yield. The grand growth is a stage when after around 120 days post-planting, sugarcane starts adding leaves, millable cane, and basal sugar accumulation and lasts up to ± 270 days post-planting. Maturation follows where there is a rapid accumulation of sugar, which is then converted to sucrose and lasts up to ± 360 days post-planting. During the two stages of tillering and grand growth up to 80% of sugar yield is produced (Gentile *et al.,* 2015). Both of these stages are known to be critically affected by water-deficit stress, which would negatively influence growth directly through dry matter accumulation and cane yield and juice quality (Khaled *et al.,* 2018). During these stages, severe drought conditions over a prolonged period of time and even moderate drought would affect the entire plant's growth and structure, mainly through stomatal limitation that result in decreased stomatal conductance, transpiration rate, internal CO_2 concentrations and photosynthetic rate (Ferreira *et al.,* 2017).

Different levels of drought tolerance have been seen in tolerant and susceptible sugarcane cultivars. Susceptible or sensitive genotypes are known to have reduced (stalk) yield (Silva *et al.,* 2008; Waclawovsky *et al.,* 2010). Tolerant genotypes physically display avoidance traits such as leaf shedding and reduced leaf area, leaf rolling and reversible early stomatal closure. Such cultivars with these reversible traits during water deficit, have a reduced CO_2 assimilation and transpiration rate, which can quickly resume upon water becoming available again. Leaf shedding and stalk senescence during extended water-deficit are a sign of a more drastic response than leaf rolling or stomatal closure because recovery from these takes time and is not guaranteed (Inman-Bamber, 2004). Sugarcane genotypes with deep root systems has also been found to tolerate the effects of drought stress better as

these root systems maintains high water absorbance from soil which allow the crop to convert available water to biomass (Smith *et al*., 2005).

1.3 GENERAL PHYSIOLOGICAL EFFECTS OF WATER DEFICIT STRESS ON PLANTS

The overall effects of drought on plants is a change in a number of morphological, physiological and biochemical processes which interfere with the configuration and function of key bio-molecules in the plant, such as nucleic acids, amino acids and proteins, carbohydrates, lipids (fatty acids), hormones (e.g ABA, auxins, ethylene, cytokinins), ions and nutrients (Boutraa *et al*., 2010). Initially, the destructive effects of drought are linked with the low osmotic potential (osmotic stress) of the soil which is a result of the reduced influx and volume of water that are being absorbed from the soil by the plant roots. The first phase of drought, namely osmotic stress triggers processes that are responsible for maintaining concentrations of cellular osmolytes and stomatal regulation, nutrient mineral and water uptake and cell turgor (Sharma *et al.,* 2012). Generally, water-deficit stress, due to constrained transpiration rates and compromised active transport and membrane permeability, reduces nutrient uptakes by the roots and the transport of these nutrients from the roots to the shoots. Reduced soil moisture content also results in diminished diffusion rates of nutrients absorbed by root surfaces from the soil (Vasantha *et al.,* 2017). When water-deficit stress persists, plants exhibits leaf rolling followed by wilting and bleaching that ultimately results in plant death (Sahoo *et al.,* 2013). In addition, plants show various damages which comprise of oxidative stress or cell injury through build-up of reactive oxygen species (ROS) and increasing cellular temperature, this in turns lead to a rise in cellular viscosity, modification in protein-protein interactions, protein clustering and breakdown (Farooq *et al.,* 2008). One of the main reasons for the excessive production of ROS under abiotic stress is the impairment of the plant's photosynthetic machinery.

1.3.1 Adjustment of photosynthesis during drought

Photosynthesis is a major primary physiological process in plants and are fundamentally affected by drought. During photosynthesis, plants uses sunlight to convert CO_2 and water to copious amounts of sugar and oxygen, which is required for cellular metabolism and in

turn support plant growth and development (Ashraf and Harris, 2013). Photosynthesis occurs in chlorophyll, which is situated in the thylakoid and forms a key component of the chloroplast, where photosynthesis is responsible for solar energy absorption and electron transport system and carbon dioxide (CO_2) reduction. During this process the energy of light is converted to ATP, NADPH and oxygen (O_2) are released into the atmosphere, while CO_2 enters through the opened stomata and are reduced to carbohydrates, ADP and $NADP^+$ (Ashraf and Harris, 2013).

The overall photosynthetic capacity of plants can be decreased in response to drought when water-deficit damage photosynthetic pigments which entail specifically the breakdown of chlorophyll and the corrosion of the thylakoid membranes (Ashraf and Harris 2013). This in turn interferes with the electron transport system and secondary oxidative damage occurs. Water-deficit stress also directly reduces rates of photosynthesis by stomatal closure and the decrease in internal carbon dioxide concentrations (Ashraf and Harris, 2013; Chaves *et al.,* 2009). Briefly, stomatal closure directly decreases the available CO_2 due to a decline in the water potential of guard cells and upsurges in gaseous carbon concentrations within the leaves and other deficiencies in the photosynthetic metabolism (Figure 1.3; Vishwakarma *et al*., 2017).

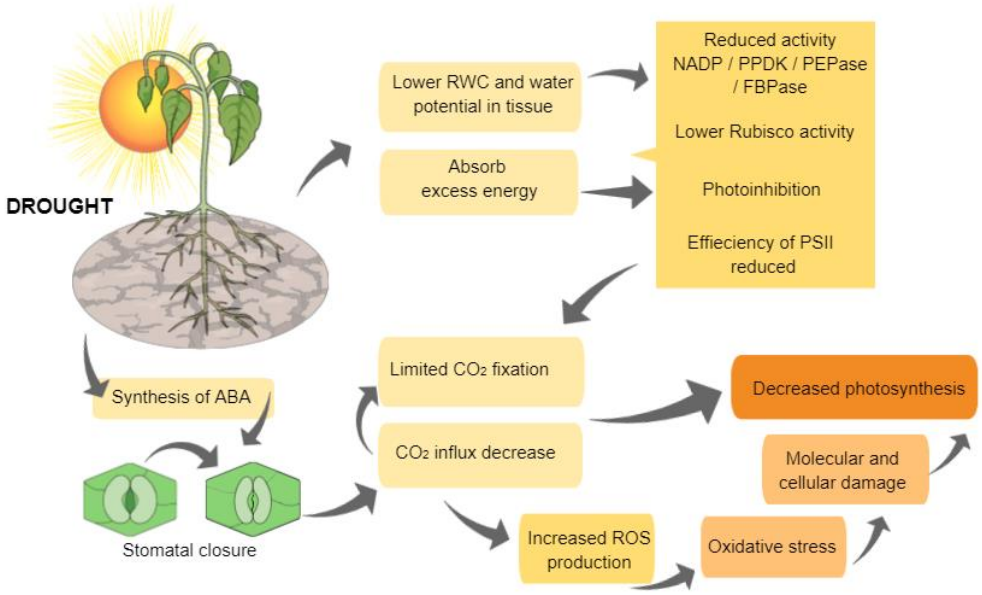

Figure 1.3. A simplified representation where drought conditions limit photosynthesis. Under water-deficit stress abscisic acid is synthesized resulting in stomatal closure and leading to a reduction in intracellular carbon dioxide concentrations which will decrease photosynthesis. Drought also reduce the water content in especially leaf tissue which influence the activity of RuBISCO and other enzymes needed to maintain carbon dioxide fixation and photosynthesis. Image created using https://mindthegraph.com software.

1.3.2 Abscisic acid influence the abiotic stress response in plants

Abscisic acid (ABA) is a plant hormone normally found in small amounts in plants grown under normal environmental conditions and was first identified in cotton leaves 60 years ago (Milborrow, 1974; Sheard and Zheng, 2009). Abscisic acid is now known to be an important phytohormone which plays a functional role in many plant development processes, such as seed and bud dormancy, organ growth and most importantly stomatal closure. It acts as a signal messenger by regulating physiological machinery which will favour adaptation and stress responses (Basu *et al.,* 2016). Some known responses include improving deeper root system growth for water uptake and maintenance of water status, decreasing transpiration rate through inhibiting shoot growth, preventing dehydration through promoting stomatal closure and facilitation of leaf senescence (Hunter *et al.,* 2004; Liu *et al*., 2003; Parent *et al.,* 2009). In addition, ABA regulates an array of genes that

coordinates cellular responses conferring plant resistance towards water-deficit stress and against pathogen attacks (Sah *et al.,* 2016; Swamy and Smith, 1999; Tuteja, 2007).

When the soil water potentially decreases or the plant is expose to a number of other stress parameters, ABA is produced in the plant roots and then swiftly translocated to the leaves by the transpiration stream in the xylem. In the leaves, ABA rapidly alters the osmotic potential of the stomatal guard cells, causing a drop in turgor pressure and the stomata to close. Stomata closure reduces transpiration, which prevent water loss from the leaves in situations where water availability is restricted (Kuromori *et al.,* 2018). Furthermore, ABA accumulation also stimulates other molecules like hydrogen peroxide (H_2O_2) as a signal mediator for anti-oxidative responses (Llanes *et al*., 2015; Xing *et al*., 2008).

Abscisic acid biosynthesis is synthesized in a complex biosynthesis pathway (Tuteja, 2007; Xiong and Zhu, 2003) through enzymatic activities, via the calcium dependant phosphorylation signalling and other cross-communicating ABA signalling factors. Calcium ions (Ca^{2+}) are a secondary messenger activated as an intracellular signal in the membrane when a stress is sensed (Figure 1.4; Tuteja, 2007). β-carotene serves as the primary source utilized by zeaxanthin epixodase (ZEP), 9-*cis*-epoxycarotenoid dioxygenase (NCED), ABA-aldehyde oxidase (AAO) and the myelobdenum cofactor sulfurase (MCSU) enzymes. In the plastid, ZEP causes oxidation of two precursors, zeaxanthin and violaxanthin to form the NCED substrate, neoxanthin, which is converted to the xanthoxin intermediate, leading to the formation and transportation to the cytosol (Figure 1.4) (Tuteja, 2007; Xiong and Zhu, 2003).

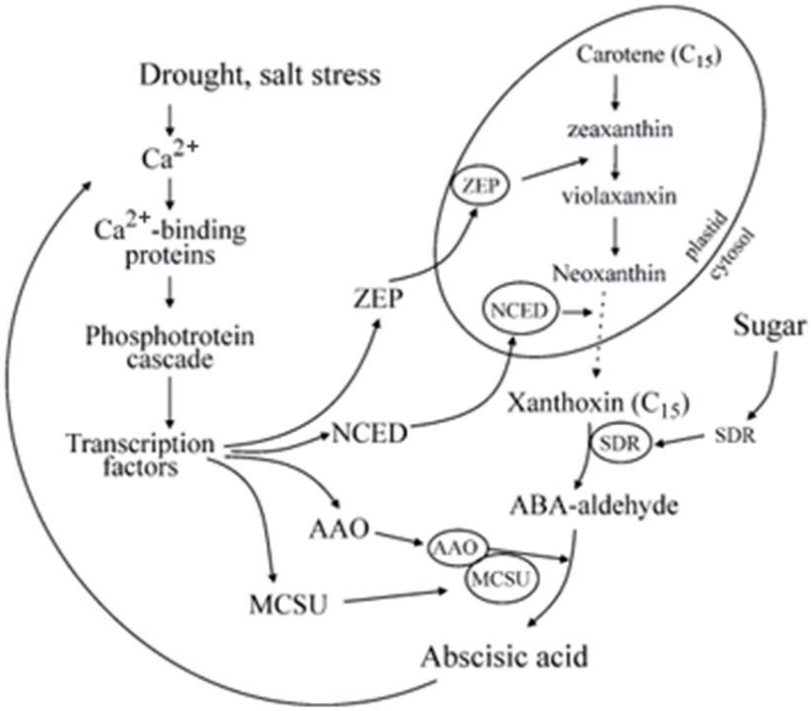

Figure 1.4. An illustration of the pathway in which ABA is synthesised from the plastid to the cytosol. Abscisic acid synthesis is initiated through the cleavage of β-carotene and the conversion to xanthoxin through an intermediate to form ABA-aldehyde (Xiong and Zhu, 2003).

Abscisic acid is perceived by PYRABACTIN RESISTANCE 1 (PYR1), or structurally similar membrane proteins, which in turn inhibits the ABI-INSENSITIVE (ABI1) phosphatase enzyme. This leads to the activation of the SNF1-related protein kinases (SnRK2), which activates several TFs from the ABA RESPONSIVE ELEMENT-BINDING FACTOR (ABF) family. ABFs alter the expression of numerous stress-response genes (Nakashima and Yamaguchi-Shinozaki, 2013). However, transcription factors (TFs) switch on stress-response genes by binding to dehydration responsive elements (DRE) and ABA-responsive elements (ABRE) though an ABA-dependant or independent signal (Chinnusamy *et al.,* 2004; Grillo *et al.,* 1995; Xiong and Zhu, 2003). Genes such as the B-box zinc finger (BBX) TFs binds to ABRE via an ABA-dependant manner.

1.3.3 Oxidative stress and Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are produced under normal respiratory conditions in plants and are a continuous by-product of cellular metabolic pathways (Gill and Tuteja, 2010). Oxygen (O_2) is continuously produced in the chloroplast during the light reactions of photosynthesis and accepts an electron through the electron transport chain, subsequently forming a superoxide (O_2^-) ion (Ashraf, 2009) or other ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical (^-OH) and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010).

Reactive oxygen species are beneficial in some metabolic processes due to their signaling roles performed in plants (Mittler *et al.,* 2006). Hydrogen peroxide, at low concentrations, acts as secondary messengers involved in providing stress tolerance by activating defense genes in response to environmental changes, regulating key physiological processes such as photosynthesis, stomatal conductance, growth and development and programmed cell death (Gill and Tuteja, 2010). Reactive oxygen species levels is controlled by maintaining an equilibrium between their production and scavenging (Apel and Hirt, 2004; Mittler *et al.,* 2006). However, excessive generation of ROS occurs during abiotic stress, which is one of the main roots of cellular damage (Ashraf, 2009). When produced at high concentrations, due to stresses perceived by the plant, ROS can oxidize/damage several components in plant cells, including proteins, lipids (lipid peroxidation) and nucleic acids, such as RNA and DNA (Ashraf, 2009).

Drought-induced ROS overproduction, can furthermore cause widespread peroxidation of membrane lipids and lead to the production of malondialdehyde (MDA) (Del Rio *et al.,* 2005). Malondialdehyde is a toxic, mutagenic and antherogenic molecule that amalgamates with DNA and proteins. It serves as a polyunsaturated precursor of lipid peroxidation and its presence is a telltale sign of free radical reactions occurring in the plant tissue (Anjum *et al.,* 2011; Gill and Tuteja, 2010) (Wang *et al.,* 2013). Malondialdehyde is formed when highly active $^{\cdot}OH$ instigate the disruption of a hydrocarbon within an unsaturated chain of polyunsaturated fatty acids. Reactive oxygen species directly attacks membrane lipids during peroxidation, which in turn causes membrane instability (Anjum *et al.,* 2011).

Similarly, as with lipid peroxidation, $^{\cdot}OH$ radicals cause replication inaccuracies and cell membrane destruction (Gill and Tuteja, 2010).

1.3.4 Enzymatic and non-enzymatic antioxidants

To remove excess ROS, plants make use of enzymatic and non-enzymatic antioxidants which act as ROS scavengers. As a result normal plant functions are stabilized and the negative effects inflicted by adverse environmental conditions are mostly eliminated (Mittler, 2002). Antioxidants include compounds such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and monodehydroascorbate (MDAR). The production of antioxidants are mainly adaptive mechanisms that have been cultivated naturally in plants to oppose the deleterious effects of drought and other related stresses on plant metabolism (Sharma *et al.,* 2012).

Superoxide dismutase is a key primary antioxidant that is highly expressed under extreme abiotic stress conditions that reacts to surplus levels of ROS within plant cells (Ashraf, 2009). Enzymatically, it detoxify the formation of H_2O_2 and ^{-}OH (Gill and Tuteja, 2010; Koyro *et al.,* 2012) by catalyzing the dismutation of O_2^{-} radicals into O_2 and H_2O (Ashraf, 2009; Gill and Tuteja, 2010). Based on the attachment of a metal cofactor, the active sites of SOD in plants are categorized into three types of isoforms, namely: i) the copper-zinc containing superoxide dismutase (Cu/Zn-SOD), which is the most prevalent in addition to the ii) manganese containing superoxide dismutase (Mn-SOD) and iii) iron containing superoxide dismutase (Fe-SOD) (Ashraf, 2009; Gill and Tuteja, 2010; Koyro *et al.,* 2012).

Glutathione (L-y-glutamyl-L-cysteinylycine, GSH) form part of the most central cellular antioxidant system. In its reduced form, GSH serves as enzyme scavenging ROS and nitrogen species, thereby contributing to the control of redox homeostasis in numerous metabolic pathways at the cellular level (Dubreuil-Maurizi and Poinssot, 2012; Noctor *et al.,* 2012). Like many cysteine comprising molecules, glutathione is readily oxidized where a disulphide bond forms between two glutathione molecules to generate the oxidized dimer (GSSG) in the presence of NADPH as an electron donor. The dimer configuration appears when ROS provide one electron to the GSH molecule, resulting in increased reactive glutathione, where it controls H_2O_2 concentrations (Couto *et al.,* 2016).

Further main H_2O_2 scavengers are APX and CAT. Catalase decompose the dismutation of two molecules of H_2O_2 to H_2O and O_2 without expending reductants and in the process present plant cells with an effective mechanism to rid cells of the constant production of H_2O_2, while APX utilizes a substrate named ascorbic acid (ASA) to reduce H_2O_2 to H_2O (Foyer and Noctor, 2000). In plants, CATs are essentially found in the glyoxysomes of lipids storage tissues where they decompose produced H_2O_2 lipid β-oxidation and peroxisomes by eliminating H_2O_2 present during photorespiration (Koyro *et al.,* 2012).

Non-enzymatic compounds acting within the antioxidant system include compatible solutes such as tocopherols, flavanones, carotenoids, anthocyanins and osmolytes (e.g. proline) (Sharma *et al.,* 2012). Compatible solutes are non-toxic molecules which can accumulate at elevated concentration without obstructing normal metabolism. Drought and other abiotic stresses results in the accumulation of compatible solutes in the cytoplasm (Bhaskaran *et al.,* 2013). In addition to their fundamental roles in regulating defense mechanism to balance water potential, compatible solutes amalgamate with various cellular components and contribute in maintaining ion homeostasis and cell turgor, stabilize some protein complexes and membranes, serve as an osmotic protectant and may interject to scavenge ROS (Bhaskaran *et al.,* 2013; Ranganayakulu *et al.,* 2013; Sharma *et al.,* 2012).

1.4 SUGARCANE IMPROVEMENT

1.4.1 Breeding strategies and its limitations

The sustainability of any global crop production is critically dependent on improving relevant crop traits, including tolerance to various types of biotic and abiotic stresses. Several approaches have been very successful towards improving sugarcane yield, focusing on traits that consistently produce high sugar yield and sugar content over the last 30 years (Cheavegatti-Gianotto *et al.,* 2011). Amongst these, breeding strategies were able to generate genotypes with high sugar content and cane yield but had limited success in protection against long-term environmental and biotic stresses (Zhou, 2013).

Traditional breeding in sugarcane faces a number of challenges which includes limited genetic variability in their gene pool, which may provide few opportunities for major step changes for further trait improvements (Richards, 1996; Tester and Bacic, 2005). In addition, in sugarcane conventional breeding efforts have been hampered due to the complexity of the genome and limitations in respect to inflorescence and pollen production (De Setta *et al.*, 2014). Furthermore, traditional breeding approaches as a whole are time consuming and labour intensive, undesirable genes are often transmitted in combination with desirable ones, and reproductive hurdles limit transfer of favourable alleles from inter-specific and inter-generic sources (Ashraf and Akram, 2009). Due to these reasons, plant biotechnology techniques such as genetic engineering could be a possible approach and have the ability to use candidate genes for targeted traits and alter key genes to produce novel phenotypes in relative shorter time periods (Scortecci *et al.,* 2012).

1.4.2 Genetic engineering of sugarcane

It is essentially accepted that traditional breeding can no longer realistically introduce novel desirable traits into commercial sugarcane cultivars and biotechnological tools will have to be used in the future to overcome this shortfall and increase the productiveness of selective breeding programs (Lakshmanan *et al.,* 2005). Therefore, genetically modified sugarcane that incorporates genes to increase resistance to biotic and abiotic stress could play a major role in the sustainable production of this crop. Genetic engineering and transgenic approaches are being employed emphatically worldwide for improving stress tolerance, quality and yield potential of many crops (Ashraf and Akram, 2009).

Since 1992, scientists were able to insert transgenes into the sugarcane genome using biolistics-mediated transformation protocols (Bower and Birch, 1992). Already, sugarcane with biotic stress resistance was generated through genetic modification. The sugarcane plant was produced to overexpress a *Bacillus thuringiensis* (Bt) *CryIAB* endotoxin, which provided immunity against some insect attacks (Arencibia *et al*., 1997; Arvinth *et al.,* 2010). Genetic engineering for increased sugar yield was achieved through the manipulation of the sucrose-1-fructosyltransferase gene (Nicholson, 2007), even though only a slight increase in total sucrose content was seen. To date, only one transgenic sugarcane genotype has been

commercially released in Indonesia (Noguera *et al*., 2015). This genotype expresses the choline dehydrogenase (*betA*) enzyme and result in a drought tolerant sugarcane line which accumulate glycine betaine, a membrane protectant. However, the use of high-throughput sequencing and functional genomics, resulted in the identification of numerous functional genes and regulatory genes involved in biological process including abiotic stress tolerance, which might be useful targets in manipulating the sugarcane genome for positive trait introductions (Singh *et al.,* 2002; Uno *et al.,* 2000).

In addition, modern genome-editing technologies, which allow targeted mutation inductions at specific DNA sequences in an organism's genome, are versatile tools for genetic engineering and open opportunities for crop improvement. Tools such as zinc finger nuclease (ZFNs) and transcription activator-like effector nucleases (TALENSs) have been used in attempts to edit crop genomes (Urnov *et al*., 2010; Wang *et al*., 2014). Only a single gene editing attempt in sugarcane has been reported where a TALEN-mediated approached was used to reduce the lignin content in the cane (Jung and Altpeter, 2016). Technologies such as the CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeat-associated nuclease 9) have been reported in food crops but not in sugarcane (Mohan, 2016; Zaman *et al*., 2019). Application of this system in sugarcane would be challenging due to the highly polyploidy and heterozygous genome of this crop species. Attempts have been made to target the endogenous acetolactate synthase gene, which when mutated convey herbicide tolerance (Oz *et al*., 2018). Genome editing without doubt has the potential to produce genetically edited crops, which will assist in attempts to ensure future sustainable productive agriculture. As editing technology is being developed and improved it might in the future be possible to perform successfully in plants with complex genomes, such as sugarcane (Augustine, 2017). However, multi gene traits, such as drought tolerance might remain a challenge when using gene editing approaches.

We know that environmental stresses cause complex physiological and biochemical responses in the plants through alterations in constitutive and specific genes expression profiles by regulatory gene expression mechanisms (Ahuja *et al.,* 2010). Genetically modifying a specific regulatory gene/s can impact plant stress tolerance by imitating stress signals to regulate several down-stream stress responsive genes in plants (Century *et al.,* 2008). Among these regulatory genes, transcription factors (TFs) are natural regulators of

cellular processes, and are potential candidates for modifying complex traits in crops through their ability to influence the expression of more than one gene possibly linked towards abiotic stress tolerance (Century *et al.,* 2008; Wang *et al.,* 2016c).

1.5 TRANSCRIPTION FACTORS (TFs)

Knowledge of the structure of TFs and their molecular mechanism are essential to understanding TFs action (Gonzalez, 2015). Transcription factors are regulatory proteins that modulate the expression of specific genes or groups of genes through recognition of a specific DNA sequence in the regulatory regions of targeted genes (Century *et al.,* 2008). Generally, TFs acts as molecular switches that activate or repress the expression of many downstream genes responsive to metabolic and environmental cues either directly, or as part of a larger protein-protein complex. They interact specifically with *cis*-elements or regulatory specific sequences located in the promoter region of the genes they regulate (Franco-Zorrila *et al.,* 2014; Joshi *et al.,* 2016; Nakashima *et al.,* 2009; Nuruzzaman *et al.,* 2013).

There are approximately, 1 500 TFs encoded by *Arabidopsis thaliana*, belonging to large families which consists of subfamilies, grouped according to two domains namely; the specific DNA binding domain and an activation domain conserved among species (Century *et al.,* 2008; Riechmann *et al.,* 2002; Wang *et al.,* 2016c). Transcription factor families are not limited to plants and some are also found in animals. Transcription factors of the same family originate from a common ancestral gene as they share similarity in structure, function and genes expressed among different plant species (Liu *et al.,* 1999).

Regulation of gene expression is critical for a variety of essential processes in plants. Basically TFs are focal regulators of gene expression modulating key aspects of plant function such as cell differentiation, tissue and organ development, responses to hormones and environmental factors, metabolic networks and diseases resistance (Gonzalez, 2015).

Gene expression towards any plant response to abiotic stress is complex and is controlled by a multitude of genes involved in stress signalling pathways. These comprises of signal perception where the stress stimuli is sensed by receptors in the cell wall that activates

secondary messengers such as ROS, cyclic nucleotides (cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)), sugars, inositol phosphate etc. (Bhargava and Sewant, 2013). These secondary messengers activate the subsequent signal transduction where phosphorylation and de-phosphorylation of proteins controlled by mitogen activated protein kinase (MAPKs) and calcium–dependant protein kinase (CDPKs) occur (Wani *et al.,* 2013; Joshi *et al.,* 2016). This is followed by the binding of the TF to a target gene followed by recruiting of RNA polymerase to activate transcription or inactivation by MAPKs/CDPKs (Joshi *et al.,* 2016; Wang *et al.,* 2016c). The signal pathway signifies that TFs are themselves regulated and subjected to several modifications at post-transcriptional level which further determines changes in the physiological and metabolic responses in the plant (Wang *et al.,* 2016c).

During the different stress response mechanisms in plants, transcriptome studies demonstrated that genes encoding transcription factors are highly regulated under drought stress. These drought responsive TFs belong to multi-gene families which mostly include the B-box zinc finger (BBX), MYB/MYC family, NAC (NAM, ATAF and CUC), dehydration-responsive element binding factors (DREB) (AP2/EREBP), basic leucine zipper (bZIP) and WRKY (pronounced “worky”) families, which is found in plants. These TFs specifically functions as downstream integrators of the regulatory networks in the expression of stress responsive genes (Gahlaut *et al.,* 2016). In recent research conducted at the IPB, in collaboration with Prof SK Panda, two TFs, *AtBBX29* and *SlNAC2* have been shown to enhance drought tolerance in model plant species (Borgohain *et al.,* 2019; Van Beek, 2018). In this study, these two TFs will be further functionally annotated with a specific focus on their potential role in water-deficit stress response in sugarcane. These two TFs belong to either the B-box zinc finger or NAC TF families.

1.6 ZINC FINGER FAMILY OF PROTEINS

The zinc finger proteins represent a family of proteins present in most eukaryotes where only the sequence and structure of the zinc finger domain are conserved across species. The zinc finger family comprises of a great number of proteins which has been grouped into multiple subfamilies. Description of these subfamilies can be found on http://smart.embl-heidelberg.de/smart/do_annotation/pl. The C2H2-type zinc finger was the first subfamily of zinc finger proteins that was classified. Represented was the zinc finger identified in the *TFIIIA* TF present in aquatic frogs by Miller *et al*. (2001) as a zinc ion binding motif with a conserved cysteine and histidine ligand. The first C2H2-type Zn finger gene that was discovered in plants was *EPF1* identified in *Petunia*, which has two C2H2 Zn finger motifs (Takatsuji *et al*., 1992).

The zinc finger domains ensemble as various finger-like protrusions on the structure of the protein that can binds metals like zinc (Laity *et al*., 2001). When acting as transcription factors, these zinc finger TFs also contain other specialized interaction domains which can bind DNA, as described in detail by the review done by Wolfe *et al*. (2000). In addition, zinc finger proteins are also now known to bind to RNA as well as other proteins (Darby, 2005; Filhol *et al*., 2005; Khanna *et al*., 2009).

Functionally, especially in relation to drought, it has been found that zinc finger proteins enhance drought tolerance in plants by increasing the levels of substances that adjust the osmotic balance, improving the ability to scavenge reactive oxygen species and increase photosynthetic efficiency in the plants (Chu *et al*., 2016a; Wang *et al*., 2016a). Transgenic rice overexpressing *ZFP252* and *Arabidopsis* overexpressing *OsMSR15*, *ZFP3, ZxZF* and *IgZFP1*, to name but a few, all showed higher levels of proline, reduced membrane damage and increased stress-responsive gene expression (Xu *et al*., 2008; Zhang *et al*., 2016a). Han and colleagues published a recent (2020) comprehensive review on the involvement of zinc finger proteins in salt, osmotic, cold, drought and high light stress. Also, zinc fingers can increase drought tolerance in plants via an ABA-dependant pathway, for example expression of *AZF2, GmZFP3*, *OsMSR15* have all been linked to ABA-related pathways under drought stress (Sakamoto *et al*., 2004; Zhang *et al*., 2016b; Zhang *et al*., 2016c).

1.6.1 Structure of the zinc finger protein

The zinc finger protrusion on the protein refers to secondary α-helix and β-sheet structures that are combined by a Zn ion (Figure 1.5). The residue of the α-helix then binds with specific DNA sequence motifs within the major groove of DNA. The binding site of the Zn ion further define the zinc finger structure into multiple (9) structural groups (Han *et al*., 2020). The majority of Zn finger proteins fall within in three groups, namely C2H2-like finger, treble-clef finger and zinc ribbon (Krishna *et al*., 2003).

The C2H2 zinc finger group has a signature cysteine/histidine motif of CX2-4CX3PX5LX2HX3-5H, where X is any amino acid, with 2 or more zinc finger domains helping to perform its signature role in sequence-specific DNA-binding (Finn *et al*., 2013). Generally, two pairs of histidines are located at the C-terminus of the α-helix and two cysteines at the end of the β-sheet bind to Zn to ensemble the tetrahedral structure (Han *et al*., 2020). The number and pattern of the zinc fingers can be further classified into fingers with a triple-C2H2 single ligand binding, multiple adjacent C2H2 ligand binding or separated C2H2 pairing. Furthermore, most C2H2-type zinc finger proteins have a conserved QALGGH motif in the Zn finger helices. C2H2 zinc finger proteins are the most common DNA-binding motifs found in eukaryotic TFs where the multiple Zn fingers can make contact along the DNA molecule and the C2H2 motifs recognise and bind to the DNA (Figure 1.5). The C2H2-type protein family consist of 176 and 189 members in *Arabidopsis* and rice respectively (Ciftci-Yilmaz and Mittler, 2008).

The treble-chef zinc fingers are best known as nuclear hormone receptors and consist of a β-hairpin and α-helix at the N and C-terminus respectively, which supply two ligands each for Zn binding. Treble clef motifs are mostly included in multi domain proteins and have additional secondary structural elements (Krishna *et al*., 2003). Zinc ribbon proteins, debatably the biggest fold group of Zn fingers, have two β-hairpins, primary and secondary, which forms two similar zinc binding sites (Gamsjaeger *et al*., 2005). The primary hairpin contains the N-terminal Zn sub-site, while the secondary hairpin contains the C-terminal Zn sub-site. Typically zinc ribbons will also have a β-sheet which forms hydrogen bonds with the hairpins (Krisna *et al*., 2003).

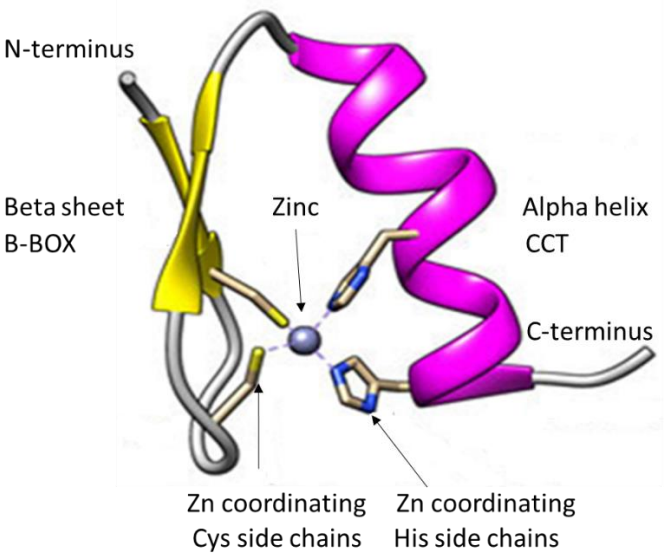

Figure 1.5. A three dimensional structure of zinc finger proteins consisting of an alpha helix and beta sheet. The Zn metal ion is coordinated by two histidine and two cysteine residues, typical of a C2H2-type zinc finger protein. The number of residues can vary and result in different fold groups of zinc finger proteins. The image is available at: https://sciencescienceeverywhere.files.wordpress.com/2015/08/figure-11.jpg.

1.6.2 The B-box zinc finger subfamily

The B-box proteins (BBX) represents one of the zinc finger protein structural classes, mostly C2H2-type, which contain a B-box domain consisting of one or two B-box motifs at the N-terminal, and sometimes a CCT (CONSTANS, CO-like or TOC1) domain at the C-terminus. The tertiary structure of the protein is stabilised by the binding of the Zn ion.

The B-box family consist of 32 proteins in *Arabidopsis* (Khanna *et al*., 2009), where the B-box motifs are typically ˜40 amino acids long, situated 5-68 and 48-130 aa from the start of the first protein residue (Yadukrishnan *et al*., 2018). In rice, Huang and colleagues (2012) identified 30 B-box genes through bioinformatics analysis of which the B-box motifs varies in length at around 47 aa at positions 1-83 and 48-149 from the first protein residue. In both plant species, 17 BBX genes contain an additional CCT domain and both *Arabidopsis* and rice *BBX* gene families are classified into five subgroups (I to V). A recent analysis of the BBX genes in other grass species identified 36 in maize, 24 in sorghum and 19 genes in millet

(Shalmani *et al.,* 2019). Overall, the B-box domain and topology were found to be conserved in a large number of proteins across the plant kingdom (Crocco and Botto, 2013).

The five B-box structural groups are described according to the presence of one (B-box1) or two (B-box2) B-box motifs and the presence/absence of the CCT domain. Groups I and II have two B-boxes in tandem, separated by 5 to 20 residues, and the CCT domain, with Group II displaying differences in their consensus sequences of the B-box2 motif (Chang *et al*., 2008). The B-box proteins of structural Group III contain a single B-box motif together with the CCT domain. Structural Group IV proteins have only the two B-box motifs, while Group V only have a single B-box domain and one amino acid less at the seventh residue position compared to the other structural groups (Khanna *et al*., 2009). In addition, in the B-box1 conserved domain of all five structural groups, the number of cysteine residues are mostly conserved. However, the B-box2 domain show low sequence identity across the Groups I, II and IV (Crocco and Botto, 2013).

1.6.3 BBX transcriptional control

B-box proteins are likely to be involved in DNA binding, RNA binding or protein-protein interactions (Gangappa *et al*., 2014). Although the biochemical characteristics of the B-box domain is not yet known, research revealed that BBX proteins especially plays an important role in transcriptional regulation in plants (Datta *et al*., 2007; Gangappa *et al.*, 2013a,b). B-box proteins can act as transcription factors or interact with other transcription factors, within or outside the BBX protein family. It is also established that these proteins can act as transcriptional co-activators but also transcription co-repressors (Gangappa *et al*., 2014). The majority of *BBX* genes that so far have been annotated, studied especially through gel-shift and one-hybrid assays in yeast (Sakamoto *et al*., 2004), possesses binding elements in their sequences allowing them to bind to the *cis*-elements in the promoter regions of downstream stress-response genes, especially in relation to abiotic and biotic stresses (Huang *et al*., 2012; Shalmani *et al*., 2019). The activation domain of B-box proteins are poorly understood but some information indicate that C2H2-type zinc finger proteins in general seem to have a proline-rich section logged amid a NLS and L-box motif, which act as an activation domain (Skakamoto *et al*., 2000). On the other hand, C2H2-type zinc finger

proteins with EAR motifs in their C-termini reduces underlying transcriptional control of other TFs, especially in regulating abiotic stress related gene expression (Han and Fu, 2019).

It is known that at least *BBX22*, *24* and *25* interact with the *HY5* (ELONGATED HYPOCOTYL 5) and the *HYH* (HOMOLOG OF HY5) bZIP-type TFs (Gangappa *et al*., 2013a, b; Sarmiento, 2013). Another BBX protein from *Arabidopsis*, encoded by the *TCR1* gene (tunicamycin-induced CONSTANS-lile-related1) is now known to interact with the *AtbZIP60* TF to counter the endoplasmic reticulum (ER) stress response resulting from unfolded proteins (Iwata *et al*., 2008). In the past, overexpression of *AtbZIP60* in cotton, pine and *Arabidopsis* lead to enhance tolerance to salt stress (Tang *et al*., 2012). Basic leucine zipper (bZIP) TFs is known for their role in regulating the expression of stress responsive genes via an ABA-dependant interaction with specific ABA-responsive *cis*-acting elements (ABRE) in the promoter region of genes (Wang *et al.,* 2016c). In apple, a number of BBX proteins play a role in the activation of the MYB transcription factors involved in fruit development (Plunkett *et al*., 2019).

1.6.4 BBX functional roles

B-box proteins play important roles in plant development and growth with specific roles in photomorphogenesis, regulation of flowering and also in responses to biotic and abiotic stresses (Crocco and Botto, 2013; Datta *et al*., 2007; Ding *et al*., 2018). A complete list of the functions assigned to the *Arabidopsis BBX* genes have been summarised by Gangappa *et al*. (2014). In addition, recent analysis of gene expression profiles implied that BBX proteins are involved in plant hormone signalling where *BBX* gene expression changed after exposure to ABA (Gangappa and Botto, 2014; Sanchez *et al.*, 2004), intercepts brassinosterioid signals (Sun *et al*., 2010), potentially play a role in gibberellin signalling (Wang *et al*., 2013; Yang *et al*., 2014; Shalmani *et al*., 2018) and was upregulated after exposure to cytokinin and auxin (Huang *et al*., 2012).

Recent research also especially link BBX proteins to abiotic stress tolerance in plants (Han *et al*., 2020). The expression profiles of numerous BBX genes in different plant species are upregulated when exposed to abiotic stresses, including drought (Cao *et al*., 2019; Chu *et al.,* 2016; Shalmani *et al*., 2019; Wei *et al*., 2020). A micro-array study conducted with a

drought tolerant versus a drought sensitive tomato line, identified a number of differentially expressed drought response BBX genes (Gong *et al.,* 2010). A number of BBX genes are also known to contain at least one known stress-responsive *cis*-element, including for example the GC-motif, ARE or the W box, making their involvement in the abiotic stress response likely (Chu *et al.*, 2016; Huang *et al*., 2012).

Two *Arabidopsis BBXs* genes, *AtBBX5* and *AtBBX24* were found to enhance osmotic stress tolerance through an ABA-dependant pathway (Nagaoka *et al*., 2003; Min *et al*., 2015). The *BBX24* and *BBX22* genes from *Chrysanthemum* increased cold/drought tolerance (Yang *et al*., 2014). *CmBBX22,* an orthologue of the *BBX22 Arabidopsis* gene, enhanced drought tolerance in transgenic plants by down-regulating senescence-associated genes and chlorophyll catabolic genes (Liu *et al*., 2019). The *BBX* genes, *VvZFPL* from grapevine protected transgenic *Arabidopsis* against cold, drought and salinity stress (Takuhara *et al.,* 2011). Overexpression of *VvZFPL* enhanced root growth under salt stress but down regulated transcription of photosynthesis-related genes and decreased chlorophyll in rosette leaves of the transgenic plants (Kobayaski *et al*., 2012). *MaCOL1* from banana was highly expressed under various abiotic stress conditions and during fruit ripening (Chen *et al*., 2012). A B-box gene isolated from apple, *MdBBX10*, enhanced tolerance to abiotic stress when overexpressed in *Arabidopsis*. It specifically enhanced the transcript levels of ABA-related genes, as well as genes linked to ROS scavenging under stress (Liu *et al*., 2019). However, although progress has been made in understanding the function of a number of BBX proteins many still needs to be functionally annotated.

1.7 NAM, ATAF, CUC (NAC) TRANSCRIPTION FACTORS

The NAC TF gene family derived from the names of the first three NAC TFs, NAM (no apical meristem) from petunia, ATAF1, ATAF2 and CUC2 (cup-shaped cotyledon) from *Arabidopsis,* which were initially discovered to have the same NAC domain (Li *et al.,* 2019; Shao *et al.,* 2015; Souer *et al.,* 1996). The NAC (NAM, ATAF, and CUC) TF family have received substantial consideration as regulators in both abiotic and biotic stress signalling pathways (Naruzzaman *et al.*, 2010: Naruzzaman *et al.,* 2013). These proteins constitute a large TF family, which are expressed in a wide range of plants species (Naruzzaman *et al.*, 2010:

Naruzzaman *et al.,* 2013). Currently, genome wide characterization has identified numerous putative NAC TFs across different species including 117 in *A. thaliana,* 152 in soybean, 151 in rice, 152 in maize and 104 in tomato (Joshi *et al.*, 2016; Le *et al.,* 2011; Naruzzaman *et al.*, 2010; Su *et al.,* 2015; Shao *et al.,* 2015; Wang *et al.,* 2016c).

1.7.1 Structure of the NAC proteins

Characteristic features of the NAC protein family members are composed of a highly conserved DNA binding NAC domain at the N-terminus region and a highly variable C-transcriptional regulation (TR) domain (Figure 1.6; Ciarmiello *et al.,* 2014; Seo and Park, 2010).

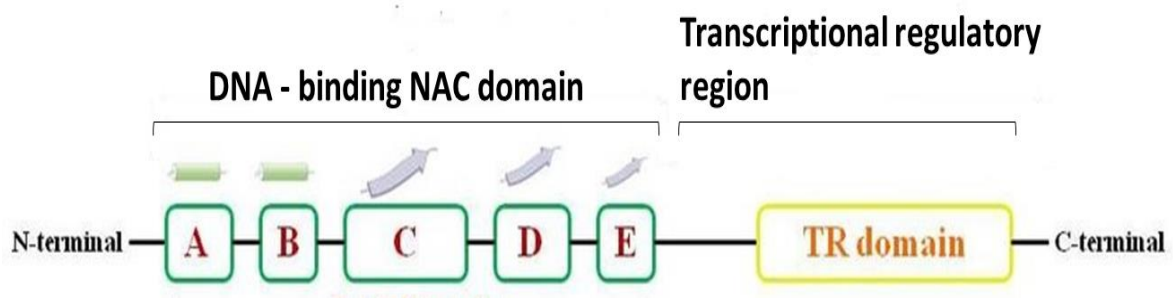

Figure 1.6. Structure of the NAC protein. The N-terminus of a NAC TF consists of subdomains (A-E) responsible for DNA binding. The C-terminus is the functional domain with transcriptional regulatory or repressor motifs (Ciarmiello *et al.,* 2014).

The N-terminus possesses approximately 150 - 160 aa linked to form the DNA binding domain, nucleus oriented localization and formation of homodimers or heterodimers with other NAC domain containing proteins (Gahlaut *et al.,* 2016; Naruzzaman *et al.,* 2010; Shao *et al.,* 2015). The N-terminus is further classified into five subdomains (A-E) (Figure 1.6; Ooka *et al.,* 2003) with subdomain E flanking the D domain, which play a role in DNA binding. The NAC A and B subdomains are rich in acidic aa and harbour a negative charge while the rest are basic and positively charged (Huang *et al.,* 2012). Crystallographic studies on the first isolated ANAC domain from *Arabidopsis* provided a completely different structure that presented a clear understanding on the function of NAC domain (Ernst *et al.,* 2004). For example, the *AtNAC019* gene structure has the ability to form a highly twisted

antiparallel β-sheet that binds to DNA as a dimer and interact with the positively charged residues at the highly conserved N-terminus (Figure 1.7) (Christianson *et al.,*2010; Ernst *et al.,* 2004; Huang *et al.,* 2012).

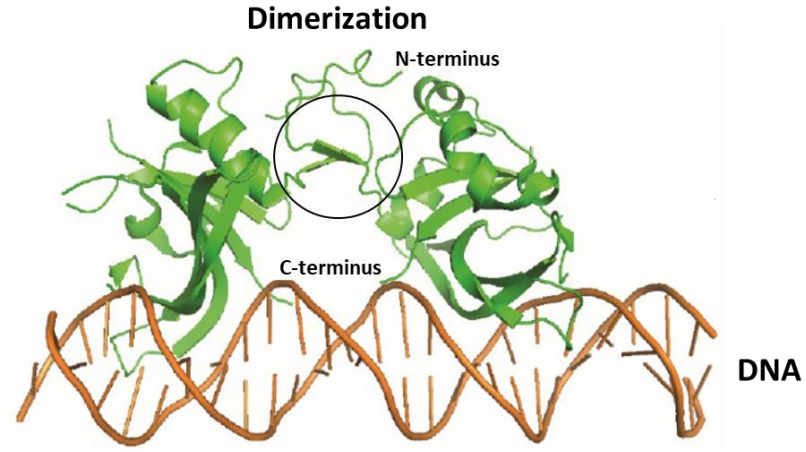

Figure 1.7. Structure of a NAC TF bound to target DNA. The NAC domain is forming a NAC-DNA complex through dimerization with the N-terminus part of another NAC domain (Gonzalez, 2015; Podzimska *et al.,* 2015).

The variable C-terminus is a functional domain responsible for transcription or repression of genes expression. The C-terminus is also responsible for the variability in transcriptional activation of NAC proteins (Gahlaut *et al.,* 2016; Naruzzaman *et al.,* 2010). Some C-terminus region of the NAC TFs contains a trans-membrane motif (TMs) and a α-helical trans-membrane motif for transcription or repression of genes (Gahlaut *et al.,* 2016; Seo *et al.,* 2008; Seo and Park, 2010).

1.7.2 Function and regulation of the NAC transcription factors

Genetics and molecular studies on NAC TFs showed that they partake in the regulation of a wide range of biological responses. These include organ development, flowering, hormone signalling, cell division, senescence, ion maintenance and pathogen defences (Breeze *et at.,* 2011; Christianson *et al.,* 2010; Kim *et al.,* 2006; Nakashima *et al.,* 2012; Olsen *et al.,* 2005; Takada *et al.,* 2001; Tran *et al.,* 2010; Sablowski and Meyerowitz, 1998; Wang *et al.,* 2016; Zhong *et al.,* 2010). For example, the SNAC genes (*OsNAC10, OsNAC5, OsNAC6, ONAC45* and *OsNAC9/SNAC1*) when overexpressed in rice resulted in root architectural modifications

including root thickening and enlargement, and higher grains yield (Hu *et al*., 2006; Joshi *et al*., 2016; Jeong *et al.,* 2010; Redillas *et al.,* 2012; Takasaki *et al.,* 2010). Overexpression of *NAC1* in rice (Stress-responsive NAC1 (SNAC1)) are associated with auxin signalling in the formation of lateral roots (Hu *et al.,* 2006; Joshi *et al.,* 2016; Liu *et al.,* 2014b; Tran *et al.,* 2004;Xie *et al.,* 2000). In pathogen defence, *Arabidopsis* ATAF1/2 was highly expressed during wounding and pathogen attack (Aida *et al.,* 1997).

In response to abiotic stresses (drought, high salt and low temp), in transgenic cotton, *SNAC1* overexpression in the guard cells reduced transpiration loss due to stomatal closure and improved drought tolerance in dry fields. Transgenic rice displayed increased germination rate, growth levels and ultimately improved tolerance to high salt conditions (Century *et al.,* 2008; Hu *et al.,* 2008; Joshi *et al.,* 2016; Liu *et al.,* 2014b; Singh *et al.,* 2015; Zheng *et al.,* 2009). *OsNAC5* expression was also reported to be induced by salinity and drought. *OsNAC5* TFs was associated as a positive regulator of stress-responsive genes. Song *et al.*, (2011) showed that a *OsNAC5* knockout using interference RNA (RNAi) resulted in transgenic rice and *Arabidopsis* which were highly sensitive to salt stress due to a downregulation of 63 stress responsive genes (Joshi *et al.,* 2016; Song *et al.,* 2011; Takasaki *et al.,* 2010; Zhang *et al.,* 2016). This shows the differentiation in NAC TFs where the overexpression or knockdown of the NAC gene may affect the expression or inactivation of stress responsive genes (Joshi *et al.,* 2016; Sakuraba *et al.,* 2015a).

Research in *A. thaliana* has indicated that the NAC proteins target five different DNA binding sites which constitute NAC regulation, some includes the drought-responsive NAC recognition sequence (NACRS; CGT(G/A) and core DNA binding sequence (CDBS; CACG) located in the promoter region of the EARLY RESPONSIVE TO DEHYDRATION1 gene (ERD1; Le *et al*., 2011; Wang *et al.,* 2016c). NAC proteins regulates the downstream target genes by interacting with these recognition sites, also called *cis*-acting elements. There is also evidence that indicates a NAC TF interaction with one or more than one *cis*-acting elements during drought stress (Gahlaut *et al.,* 2016 Wang *et al.,* 2016c), which includes *cis*-elements such as the ABA responsive element (ABRE), dehydration responsive elements (DRE), salicylic acid responsive element and jasmonic acid responsive element (Nakashima *et al.,* 2012). Transgenic *Arabidopsis* expressing three ANAC genes (*ANAC019, ANAC055* and/or *ANAC072*) upregulated drought responsive gene expression by recognising or targeting the

ERD1 promoter under water-deficit conditions and enhance drought tolerance in these plants (Nuruzzaman *et al.,* 2013). In addition, the *AtNAC3* gene, which was found to bind to the ERD1 promoter improve tolerance to severe drought stress (Hu *et al.,* 2006; Joshi *et al.,* 2016; Liu *et al.,* 2014a; Tran *et al.,* 2004).

Protein-protein interaction of NAC proteins involves dimerization, where NAC to NAC proteins interacts and forms a homodimer or heterodimer with other TF family proteins to produce a functional DNA binding TF (Figure 1.6; Gonzalez, 2015). For example, *ANAC096* and *ANAC072* expression was linked with interacting with the ABRE binding factor of the basic leucine zipper (bZIP) protein and synergistically activates ABA-inducible genes such as *RD29A* and *RD26* in dehydrated plants or under osmotic stress (Peng *et al.,* 2015). Likewise, *ANAC019, ANAC055* and *ANAC072* proteins were found to interact with a zinc finger homeodomain 1 (*ZFHD1*) DNA binding domain which synergistically activated the (EARLY REPSONSIVE TO DEHYDRATION 1) *ERD1* promoter (Gonzalez, 2015).

Signifying the transcriptional activity of NAC TF further, a study conducted by Huang *et al.* (2015) and Zhang *et al.* (2016b) overexpressed three wheat NAC genes, namely *TaNAC47, TaNAC67* and *TaNAC29* in *Arabidopsis* resulting in enhanced tolerance to drought stress in transgenic plants which displayed a high germination rate and enhanced root growth in addition to less cellular damage, hydrogen peroxide (H_2O_2) and high antioxidant activity. *TaNAC47* transgenic *Arabidopsis* also had increased soluble sugars and proline content, which contributed to the adjustment to osmotic stress (Huang *et al.,* 2015; Zhang *et al.,* 2016b). These transgenic plants overexpressed six stress responsive genes, namely *RDA29A, RDA29B, COR47, RD20, GSTF6 and P5CS1* (Jiang *et al.,* 2014), which amongst other functions, are involved in proline synthesis and cellular dehydration. The same was seen in transgenic plants overexpressing the *TaNAC67* gene, resulting in elevated expression of the *DREBI1A, RDA29A, RDA29B, RAB18* and *AB15* stress-responsive genes. *ABI5* is for example involved in ABA signalling and stress response. All these genes carry a NAC-binding *cis*-element in their promoter regions and are therefore transcriptionally regulated by NAC TFs (Jiang *et al.,* 2014; Mao *et al*., 2014; Zhang *et al.,* 2016b).

Furthermore, *TaNAC29* regulates ABA signalling and reduced expression of *RD29A, SAG13, SAG113, AIB1, ERD11*, and *AB15* genes (Gahlaut *et al.,* 2016; Huang *et al.,* 2015).

SENESCENCE ASSOCIATED GENES (SAG) as known to be involved in the regulation of processes associated with senescence (Sarwat and Tuteja, 2018). A comprehensive list of functionally annotated NAC TF genes linked to drought tolerance in plants are listed in the table below (Table 1.1).

Table 1.1 NAC transcription factors which were identified in a variety of crops and have been associated with regulation of stress responsive genes to enhance tolerance to drought.

NAC gene	Plant Origin	Function	Genes regulated	Reference
TaNAC47	Wheat	Proline biosynthesis, ROS detoxification	*AtRD29A/B, AtCOR47, AtRD20, AtGSTF6, AtP5CS1*	Zhang *et al.*, 2016b
CUC2	*Arabidopsis*	Embryo development, flowering	*NAP;* which targets *AP3/PI*	Sablowski and Meyerowski, 1998
OcNAP	Rice	Regulates senescence	*ANAC003, ANAC019, ANAC003, ANAC019 Speedy hynastic growth* (*SHYG), ANAC048, ANAC055, ANAC056, NARS1, RD26, ANAC072, ANAC074, ANAC079/80* and *ANAC100, ANAC041, ANAC054 (CUC), ANAC084* and *ANAC083 (VN12)*	Balazadeh *et al.,* 2010; Rauf *et al.,* 2013; Podzimska-Sroka *et al*., 2015
NON-RIPENING (NOR)	Tomato	Fruit ripening, leaf senescence	*SAG*-GENES: *SISAG15/113, SISAGR1, SIYLS4,SIKFB20, SISARG1, YSL4 (YELLOW LEAF SPECIFIC4), SIERT1b, SIADH2 (ALCOHOL DEHYDROGENASE2), SIACGS2 (1-AMINICYCLOPROPANE-1-CARBOXYLATE SYNTHASE2*	Alseekh *et al.,* 2015; Ma *et al.,* 2019; Speirs *et al*., 1998; Oeller *et al.,* 1991; Qin *et al*., 2012

SINAP2	Tomato	Delay senescence, control ABA biosynthesis	*SISAG113* (SENESCENCE ASSOCIATED GENES113), *SISGR1 (SENESCENCE-INDUCIBLE CHLOROPLAST STAY GREEN PROTEIN1*)	Ma *et al*., 2019
JUBGBRUNNENN1 (*JUB1*) also known as *NAC042*	*Arabidopsis*	Stress tolerance through signaling H_2O_2 levels	Dehydration responsive element binding protein2A (*DREB2A*) which then regulates Heat-shock factor A2; *Hsf2;* Heat-shock protein (HSP) and downregulates; protein (*HSP*; GA) and brassinosteroids (G) B proteins	Shahnejat-Bushehri *et al.,* 2016, ; Sakuma *et al.,* 2006; Schramn *et al*., 2008; Wu *et al*., 2012

Looking at available literature, it is clear that at least some BBX and NAC TFs proteins play a role in the abiotic stress response in plants. However, extensive research is required to advance our understanding of the functions and mechanisms of individual TF proteins. In the next few years unravelling the molecular mechanisms of each individual BBX and NAC protein will be a valuable undertaking. Significant progress has been made to determine the function of a large cohort of the BBX and NAC TF proteins in especially model plants species, such as *Arabidopsis,* and a few select crops and ornamental plants. To the author's knowledge, no analysis of the BBX and NAC TF protein families in sugarcane have been done so far. Biotechnology approaches, such as genetic engineering allow us to determine the functions of genes by assessing the phenotype caused by the additions of a transgene. By overexpressing individual TFs in model and ultimately crop species, we will enhance our understanding of their unique roles in improving plant tolerance and help establish strategies to counter abiotic stresses experienced by especially crop plants to ensure sustainable agricultural production under increasingly harsh environmental conditions.

CHAPTER 2: OVEREXPRESSION OF A B-BOX ZINC FINGER TRANSCRIPTION FACTOR IN SUGARCANE TO ENHANCE DROUGHT TOLERANCE

2.1 INTRODUCTION

Sugarcane (*Saccharum spp.* hybrid) is one of the most important crops in the world, grown mostly as a source for sucrose and ethanol, used as a renewable energy resource. Sugarcane is a very productive but highly water demanding crop and its growth and sugar content are restricted by drought (Kumar *et al.,* 2014). Generally, drought induce osmotic and oxidative stress in plants which triggers cellular damages, cause growth and water status interferences, changes in ion transport and nutrients uptake systems (Abbas *et al.,* 2014; Khan and Khan, 2019). Global climate changes are causing frequent and severe drought spells in southern Africa, especially prevalent over the past decade and as currently experience in large parts of South Africa (Nhamo *et al.,* 2019). Therefore, sustainable production, through improvements of sugar quality and induced tolerance towards limited water resources, are of significant importance for this crop's continues cultivation in southern Africa (Kumar *et al.,* 2014).

Due to the complexity of the sugarcane genome, which makes trait improvements through conventional breeding challenging, the alternative use of genetic engineering may contribute towards sustainable production of this crop (Butterfield *et al.,* 2002; Hussain *et al.,* 2012). Stress tolerance in plants is highly complex and involves differential expression of genes and protein-protein interaction in signaling pathways related to stress responses (Li *et al.,* 2016). Regulatory genes, such as transcription factors (TFs), are linked to abiotic stress responses in plants and can act as molecular switches for stress-response genes in plants (Gahlaut *et al.,* 2016). Genetic engineering can target these regulatory genes in order to potentially generate drought tolerant sugarcane varieties and also contribute to enhancing our understanding of the linked pathways in plant abiotic stress tolerance (Butterfield *et al.,* 2002; Capell *et al.,* 2004).

In response to water deficit, plants defend themselves through the expression of numerous stress-related genes permitting survival features and improving tolerance (Bresta *et al.,* 2018). Such changes are mostly linked to the phytohormone, abscisic acid (ABA), which are synthesized when water resources are limited (Yamaguchi-Shinozaki, 2005). Increase levels of ABA in turn mostly trigger genes responsible for physiological adaption towards drought (Xiong *et al.,* 2002). These stress-inducible target genes comprise the *cis*-acting sequences of ABA-responsive elements (ABREs) in their promoters, resulting in regulatory mechanisms that are ABA-dependent. In some instances, the B-box (BBX) zinc finger transcription factor gene family forms part of this ABA-dependent pathway, which can influence plant growth and developmental processes and contribute to abiotic stress responses through direct or indirect transcriptional regulation (Khanna *et al*., 2009; Kumagai *et al*., 2008).

B-box zinc finger (BBX) proteins represents a subgroup of the zinc finger protein family, characterized by a conserved B-box domain with one or two B-box motifs at the N-terminus in addition to the sometime present CCT (CONSTANS, CO-like, TOC1) domain at the C-terminus (Robson *et al*., 2001). The B-box motifs are composed of ~40 amino acids in length and are divided into two types, B-box1 and B-box2, based on their consensus sequence and the spacing of the zinc-finger binding domain (Gangappa and Botto, 2014). A total of 32 BBX proteins have been identified in *Arabidopsis thaliana* (Khanna *et al.,* 2009), which are simplified in five clusters (I-V) according to their B-box and a CCT domains. Of these proteins, 21 (BBX1-13 and 18-25) and 11 (BBX14-17 and 26-32) contain 2 or only 1 B-box, respectively (Gangappa and Botto, 2014), while 17 proteins (BBX1-17) have a CCT domain.

Functionally, BBX proteins have been linked to regulatory networks controlling plant development and growth, photoperiodic regulation, circadian rhythm and responses to biotic and abiotic stresses. However, the role of B-box zinc finger TFs in plant stress are still poorly understood. In a review compiled by Gangappa and Botto (2014), 13 of the 32 *Arabidopsis* BBX genes were linked to an abiotic stress response, mostly cold and salt stress. The majority of these genes also responded to an ABA input signal leading to increased expression.

B-box 29 (BBX29) is a B-box zinc finger protein that has not been fully functionally annotated. It has a single B-box domain coordinated at amino acid position 6 to 42 (Khanna *et al.*, 2009). Putative functions such as flowering regulation, since it is a member of the CONSTANS gene family, has been assigned to this gene (Putterill *et al*., 1995). *CmBBX29*, a homolog isolated from chrysanthemum, has also been linked to the regulation of flowering when overexpressed in *Arabidopsis* (Chen *et al*., 2019). One study, conducted by Mikkelsen and Thomashow (2009) identified this gene, which they named CONSTANS-like (COL1), as being upregulated by low temperature in a CBF-independent manner. Further investigations revealed an evening element (EE) motif (AATATCT), a MYB TF CCA1 binding site (AAAAATCT) and six ABRE-like motifs, one of which is part of a G-box (CACGTG), in the promoter of this gene. The G-box has been implicated in a number of ABA-regulated gene expression pathways, which include the binding of bZIP TFs (basic region/leucine zipper motif).

In this study, the *AtBBX29* gene from *Arabidopsis*, was overexpressed in sugarcane to potentially enhance drought tolerance in this crop species. This gene was identified and isolated in the laboratory of our collaborator Prof SK. Panda, Assam University, India and showed, in preliminary analysis conducted in our lab, to enhance osmotic stress tolerance in a model plant.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Chemicals used in this study were mainly purchased from Sigma Aldrich (St. Louis, USA) unless otherwise stated. Restriction enzymes and *Taq* polymerase were purchased from New England Biolabs ([NEB], Ipswich, USA). Macro- and micro-nutrients and hormones for *in vitro* plant cell growth were all obtained from Sigma Aldrich and Duchefa Biochemie (Haarlem, Nederlands).

2.2.2 Transgene

The *BBX29* TF transgene (accession number: At5g54470) isolated from *Arabidopsis thaliana* and cloned into the pJet1.2 cloning vector was provided by Prof SK. Panda, Assam University, India.

2.2.3 Standard molecular techniques

2.2.3.1 Plasmid DNA transformation and isolation

Escherichia coli bacterial cells were transformed with recombinant vector DNA using a standard heat-shock method. Briefly, competent DH5α cells were heat-shocked at 42°C for 45 sec and incubated for 1 h in liquid LB (Lysogeny Broth) medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl; pH 7) at 37°C on a shaker at 225 rpm. Transformed cells were streaked out on solid LB medium, solidified with the addition of 15 g/L agar, containing 50 mg/ml ampicillin, and incubated at 37°C overnight. Positively transformed single colonies were cultured under selection in 5 ml liquid LB medium and recombinant vector DNA was isolated from these cultures using the GeneElute Plasmid Mini-prep kit (Promega, Madison, USA).

2.2.3.2 Heat-shock competent cells

E. coli strain DH5α bacterial cells obtained from -80°C stored glycerol stocks were streak-plated on LB media and incubated at 37°C for 24 h. Starter cultures were prepared by inoculating a single colony in 3 ml liquid LB medium and placed at 37°C shacking at 225 rpm overnight. The overnight culture was sub-cultured into 50 ml LB medium, shaking at 225 rpm at 37°C, until an OD_{600} = 0.5 - 1.0 was reached. Cells were placed on ice for 15 min following centrifugation at 4000 g for 10 min at 4°C. The bacterial pellet was re-suspended in 100 ml of ice-cold 0.1 M $CaCl_2$. The cells were then placed on ice for 10 min, and centrifuged (4000 g, 10 min at 4°C). The supernatant was discarded and the pellet was re-suspended in 3.3 ml ice-cold 0.1 M $CaCl_2$ containing 15% (v/v) glycerol. Cells were then aliquot (50 µl), flash-frozen in liquid nitrogen and transferred to the -80°C freezer till used.

2.2.3.3 Electro-competent bacterial cells

The *Agrobacterium tumefaciens*, strain LBA4404, obtained from an -80°C glycerol stock was streak-plated onto YEP media (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar; pH 7) containing 50 mg/ml rifampicin and incubated at 28°C for 2 days. A single colony was inoculated into 25 ml liquid YEP medium supplemented with rifampicin and incubated at 28°C, shaking at 225 rpm until the OD_{600} reaches 0.5 - 0.7. Cultures were then placed on ice for 15 min and centrifuged in pre-cooled 50 ml falcon tubes at 5000 g at 4°C for 10 min. The supernatant was discarded and the pellet was re-suspended in 5 ml 10% (v/v) glycerol, pellet re-suspension was repeated three times after centrifugation. For the last step, the pellet was re-suspended in the residual 1.5 ml 10% (v/v) glycerol and cells were aliquot (100 µl), flash-frozen in liquid nitrogen and placed in a -80°C freezer till used.

2.2.3.4 Primer design and PCR amplification

All primers used in this study were designed using an online program, Primer 3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were synthesized and obtained from Inqaba Biotechnologies (Pretoria, South Africa). All primers linked to the *AtBBX29* transgene are listed in Table 2.1.

Polymerase chain reaction (PCR) amplification which required a proof reading functionality were conducted using Q5 High - Fidelity DNA Polymerase (New England Biolabs (NEB), Ipswich, USA) according to the manufactures instruction. Standard PCR conditions were set as follows: initial denaturation 98°C for 30 s followed by 35 cycles of denaturing at 98°C for 10 s, primer annealing between 55°C and 65°C, depending on the primer pair, extension for 40 s at 72°C and a final extension at 72°C for 2 min, followed by a decrease in temperature to 4°C.

All standard PCR screening was done using GoTaq Polymerase (Promega, Madison, USA) according to the manufacture protocol. PCR conditions were set as follows: initial denaturation at 95°C for 3 min followed by 30 cycles of denaturing at 95°C for 30 s, primer annealing between 55°C to 65°C for 40 s and 72°C for 30 s, with a final extension at 72°C for

5 min followed by a decrease in temperature to 4°C. In general, between 20 and 40 ng of plasmid DNA was used as template in all PCR reactions.

PCR amplicons were separated on a 1% (w/v) agarose TBE (5.4 g/L Tris-base, 2.75 g/L boric acid and 0.465 g/L ethylenediaminetetraacetic acid (EDTA); pH 8) agarose gel, which were stained with 0.5 µg/ml ethidium bromide and visualized using a G:BOX gel documentation system (Syngene, SDI Group, Cambridge, UK). If required, the amplicon was purified using the Wizard® SV Gel and PCR Clean-Up Kit (Promega, Madison, USA) as per instruction of the manufacture.

2.2.3.5 Genomic DNA extraction

Plant tissue, mostly leaf material at around 100 mg, was harvested and ground to a fine powder using liquid nitrogen. Genomic DNA was extracted using the ZR/Plant/Seed DNA MiniPep Kit (Zymo research, USA) according to the instructions provided by the manufacturer. DNA concentrations were determined using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific, Wilmington, USA) based on the absorbance measured at 260 nm and stored at -20°C. Genomic DNA concentrations between 200-300 ng, were used as template in PCR analysis.

2.2.3.6 RNA extraction and cDNA synthesis

Total RNA was extracted from plant tissue (100 mg) using a Maxwell® 16 LEV Plant RNA Kit (Promega, Madison, USA) following the manufacturer's protocol. For this, leaf tissue was harvested and ground to a fine powder using liquid nitrogen. cDNA was then synthesized from 1 µg RNA using the Reverse Aid H minus First strand cDNA synthesis Kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. Harvested tissue, RNA and cDNA were stored at -80°C till used.

2.2.4 Construction of a plant expression vector

The full length *AtBBX29* gene was PCR amplified using the Q5 High - Fidelity DNA Polymerase from pJet1.2:*BBX29* vector template using the BBXBamHI Forw and Rev primer

set (Table 2.1). The 600 bp amplicon was digested with *Bam*HI (NEB, CutSmart buffer, 37^{o}C) and cloned into the pUBi510 plant expression vector, which contain an ubiquitin promoter and cauliflower mosaic virus (CaMV) terminator.

For this, the pUBi510 vector was linearized by restriction digest using *Bam*HI-HF (NEB, CutSmart buffer, 37^{o}C), heat inactivated at 80^{o}C for 10 min and purified using the Wizard® SV Gel and PCR Clean-Up Kit (Promega, Madison, USA), according to the manufacturer's protocol. The purified PCR amplicon was then ligated, using T4 DNA ligase (NEB, room temperature), into the unique *Bam*HI site of the pUBi510 vector (ratio 3:1 insert/vector) creating pUBi510:*AtBBX29*. The recombinant vector was transformed using the standard heat-shock protocol into *E. coli cells* described in section 2.2.3.1 and selected on LB medium containing 50 mg/L ampicillin. Colonies were screened by PCR using GoTaq and a combination of transgene specific and vector primers to confirm gene insertion (Table 2.1). Transgene orientation and integrity was confirmed by sequencing the clones at the Central Analytic Facility (CAF) at Stellenbosch University. The Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) database or the Arabidopsis Information Resource (TAIR) database was used to analyze all sequences.

Table 2.1 PCR primers used for the cloning and screening of the *AtBBX29* gene.

Primer name	Primer sequence (5'- 3')	Function	Tm	Amplicon size
BBXBamHI Forw	GCAGGTCGACGGATCATGGGGSSGAAGAAGT GCGAGTT	Gene amplification	60°C	600 bp
BBXBamHI Rev	GAATTCCCGGGGATCTCAATAAAACGAAGACG ACGATGA			
BBXNcoI Forw	GGACTCTTGACCATGGATGGGGAAGAAGAAG TGCG	Gene amplification	59°C	600 bp
BBXNcoI Rev	CGTCGTCTTCGTTTTATTCCATGGTAGATCTGA CTACTACATA			
BBX RT Forw	CTAATTTTCTGGTGGCGAAACACATGCG	Confirm gene insertion; expression	68°C	600 bp
BBX RT Rev	CAATGGTCTAGATTGGTTCTCCTCCTGCATTTC			
Ubiquitin promoter Forw	ATACGCTATTTATTTGCTTGG	Gene orientation; vector confirmation	54°C	
Actin Forw	TCACACTTTCTACAATGAGCT	Reference gene expression; RNA quality	53°C	600 bp
Actin Rev	GATATCCACATC ACACTTCAT			
GFP Forw	AGATGGTGATGTTAATGGGC	Reporter gene confirmation	55°C	562 bp
GFP Rev	CCATTACCTGTCCACACAATCT			

2.2.5 Plant transformation, selection and regeneration

Embryogenic callus was initiated from *Saccharum* spp. hybrid, cultivar NCo310 immature apical inner leaf roll segments of sugarcane stalk material. Leaf rolls were cut into segments, between 2 - 3 mm thick and placed on MSC3 medium (4.43 g/L MS (Murashige and Skoog, 1962; with vitamins), 20 g/L sucrose, 0.5 g/L casein, 3 mg/L 2,4-D and 2.22 g/L Gelrite™; pH 5.8). Cultures were incubated in the dark at 26°C and sub-cultured onto fresh media every 2 weeks.

Prior to bombardment, as described by Birch and Bower (1994), embryogenic callus was placed in a circle (~2.5 cm) on an osmoticum medium (MSC3Osm), consisting of the basic MSC3 medium with the addition of 0.2 M of each mannitol and sorbitol, at 26°C for 4 h in the dark.

A DNA precipitation mix was prepared containing 5 mg tungsten (Grade M10: Biorad, #165-2266) sterilized in 100% ethanol and rinsed 3 times with sterile water. The tungsten was mixed with 5 µl each of the pUBi510:*AtBBX29* (1 µg/µl) and pEmuKN (1 µg/µl; selection vector), 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine and kept on ice for 5 min. The pEmuKN selection vector contained the *neomycin phosphotransferaseII* (*nptII*) gene as selection marker under control of the Emu synthetic promoter (Last *et al*., 1991).

Biolistic particle delivery was done with a custom-made system, developed at the Institute for Plant Biotechnology, Stellenbosch University. For bombardment, 5 µl of the DNA precipitation mix was placed onto the centre of a 1 mm^2 metal grid above the explant. Target tissue was placed 16.5 cm below the particle source and the helium bombardment pressure set at 1000 kPa. The vacuum chamber was evacuated to reach 80 kPa before the precipitation mix was discharged. Bombarded tissue was retained on MSC3Osm medium in the dark for 4 h. Cultures were then transferred to MSC3 medium containing 45 mg/L geneticin for 7-8 weeks in the dark at 26^{o}C with subculturing onto fresh MSC3-G45 medium every 2 weeks. After the selection phase, putative transformed calli were transferred to MSC media (MSC3 without 2,4-D) and incubated at 26^{o}C at a 16/8-hour light/dark photoperiod (50 µmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation) under cool white fluorescent lights (Osram, L 58V/740) for somatic embryo formation. Regenerated plantlets were allowed to grow to a height of ~5 cm and developed roots prior to transfer to the glasshouse.

In vitro plantlets were transferred to pots containing a mix of 1 part potting soil and 1 part sand and placed in the glasshouse at 26 ± 2^{o}C under natural light. Plants were initially covered with plastic bags to maintain a high humidity and prevent plantlets from drying out during the hardening off phase. Plants were watered every two days, and fertilized with 3 g/L Hydrotech Generic Fertilizer (Hydrotech, Stellenbosch, SA) and 2.5 g/L $Ca_2(NO_3)^2$ every two weeks.

2.2.6 Molecular analysis of putative transformed plantlets

2.2.6.1 Transgene integration

Genomic DNA from each putative transformed plantlet and non-transgenic wild-type sugarcane was extracted from 150 mg leaf tissue as previously described. Transgene integration was confirmed through standard PCR amplification using GoTaq DNA polymerase and 200 ng of gDNA as template in combination with gene specific and ubiquitin promoter pairs (Table 2.1). Amplicons were separated and visualized through gel electrophoresis.

2.2.6.2 Transgene expression

Semi-quantitative RT-PCR was performed using cDNA (1 µl) template from transgenic and wild-type plant lines to access relative levels of transgene expression. Transgene specific primers and primers designated for the *actin* gene (Table 2.1), which was used as internal reference gene, were used in combination with GoTaq DNA polymerase in standard PCRs. The 500 bp amplicon was separated on a 1% (w/v) agarose gel and visualized under UV light.

2.2.7 Analysis of plant phenotype under normal environmental conditions

Hardened off transgenic and wild-type sugarcane were transferred and grown in big 56 cm pots containing a 2:1 mix of potting soil and sand in a growth tunnel under natural light and temperature on Welgevallen Experimental farm, Stellenbosch University, Stellenbosch. The plants were regularly watered, received fertilizer during the watering regime and were allowed to grow for 8 months.

2.2.7.1 Plant growth

Agronomic growth measurements of plant height, leaf width, leaf length and internode lengths were taken from 3 stalks of each of the four (4) biological repeats for each transgenic and wild-type (WT) line. Plant height was measured from the soil level to the top visible dewlap leaf (TVD). The TVD is the first leaf from the top of the plant that has a visible dewlap formed on the stalk. The TVD was also used as measuring point for the leaf width,

which was recorded 10 cm away from the dewlap following the length of the leaf. The internode length was measured for internodes 7, 8 and 9, internodes were counted from the top to the bottom of the stalk, youngest to oldest, respectively.

2.2.7.2 Carbohydrate content

To determine the carbohydrate content, mature (internode 9) and immature (internode 3) internode tissue were harvested from the 8 months old transgenic and wild-type sugarcane plants. Stalk tissue was flash frozen in liquid nitrogen and stored at –80^{o}C till used.

Total soluble carbohydrates were extracted using the ethanol extraction method described by Chow and Landhäusser (2004). Sugars were extracted from 20 mg of ground frozen internode tissue. The tissue was homogenized in 250 µl of 80% (v/v) ethanol. The homogenate was heated at 95^{o}C while shaking vigorously for 30 min followed by centrifugation at 5 000 g for 10 min. This extraction step was repeated 3 times with the supernatant being collected after each centrifugation step. For the last extraction the ethanol concentration was lowered to 50% (v/v). The collected supernatants were combined and kept at –20^{o}C until measurements were performed.

Sugar levels were measured using the BOEHRINGER MANNHEM/R-BIOPHARM Enzymatic BioAnalysis/Food Analysis kit (R-Bopharm, Boehringer Mannheim, Darmstadt, Germany) following the manufacturer's instructions. Absorbance was measured at a wavelength of 340 nm using a VersaMax ELISA Microplate reader and the sucrose, glucose and fructose concentration were expressed as µmol/g FW.

2.2.8 Drought pot trials

Transgenic and wild-type sugarcane were multiplied *in vitro* on half strength MS (½ MS) medium containing 2% sucrose, 2 mg/l 1-naphthalene-acetic acid (NAA) and 0.22 g/l gelrite; pH 5.8. Rooted *in vitro* plantlets were harden off in the glasshouse by planting in 20 cm pots containing a homogenous mixture of soil consisting of 2:1:1 palm peat:sand:vermiculite and placed in the glasshouse at 26 ± 2^{o}C under high humidity. Plants were watered every two

days, and fertilized with 3 g/L Hydrotech Generic Fertilizer (Hydrotech, Stellenbosch, SA) and 2.5 g/L calcium nitrate [$(Ca_2(NO_3)^2$] every two weeks.

Four months old healthy plants, 25 repeats for each genotype in the tillering stage of development, were deprived of water and subjected to drought for 21 days. Once water was withheld, physiological measurements were taken on days 0, 3, 5, 7, 10, 14, 17, 19 and 21 without watering [ww] using the top visible dew lap leaf (TVD) as measuring point. In addition, leaf material above and below the TVD were harvested on days 0, 7, 14 and 21 ww, flash frozen in liquid nitrogen and stored at - 80^{o}C for further biochemical analysis. Also, during the water-deficit stress period plant growth was recorded by measuring the plant height and length and width of the TVD of the transgenic and wild-type sugarcane plants included in the trial. Measurements were recorded every 7 days on days 0, 7, 14 and 21 ww.

The soil moisture content of all pots included in the trial was measured every two days using the ProCheck probe (Decan Devices, Washington, USA) inserted 9 cm deep into the soil and recorded three readings per pot around the plant stem.

2.2.9 Physiological and biochemical analysis of plants exposed to drought

2.2.9.1 Relative water content (RWC)

A 1 cm^2 length leaf disc was cut from the TVD and fresh weight (FW) was recorded. The leaf disks were floated on distilled water at room temperature on a shaker for 24 h, dried to remove excess water, and the full turgid weight (TW) recorded. The leaf disks were then dried at 80^{o}C for 2 days and the dry weight (DW) recorded. According to Smart and Bingham (1974), RWC was calculated as a percentage using the formula:

RWC % = [(FW – DW)/ (TW – DW)] x 100%

2.2.9.2 Stomatal conductance

Stomatal conductance was measured at three surface positions of the TVD of four plants per transgenic line and wild-type sugarcane using the Decan Leaf Porometer SC-1 (Decagon Devices, Pullman, USA), according to the method described by Pask *et al.* (2012).

Measurements were taken every two days, starting with non-stress plants on day 0 ww, of the trial and expressed as mmol/m^2s.

2.2.9.3 Chlorophyll fluorescence

Chlorophyll fluorescence was measured using the OS-30p+ (OPTI-SCIENCES, Hudson, USA) chlorophyll flourometer. Following the method described by Maxwell and Johnson (2000), measurements were taken at three surface positions of the TVD of four plants from each genotype at the onset of the water stress period (day 0 ww) and again on days 3, 5, 7, 10, 14, 17, 19 and 21 ww. Clip shutters for dark adaption were applied to leaves 20 min prior to taking readings. Chlorophyll fluorescence were expressed as Fv/Fm.

2.2.9.4 Chlorophyll content

Chlorophyll was extracted according to the method described by Hiscox and Israelstam (1979). Hundred mg of grinded frozen plant tissue was homogenized in 500 µl of 80% (v/v) acetone, heated at 65^{o}C for 30 min and the supernatant was collected and kept on ice. The extraction was repeated three times and all collected supernatant phases were combined at the end of the repeated extraction procedure. The absorbance of the supernatant was measured at wavelengths 663 and 645 nm according to Arnon (1949). Total chlorophyll content was calculated using the formula:

Total chlorophyll (µg/ml) = 20.2 (A_{645}) + 80.2 (A_{663}), where:

- A_{645} = Absorbance at 645 nm
- A_{663} = Absorbance at 663 nm

2.2.9.5 Electrolyte leakage (EL)

Electrolyte leakage was measured using the AD31 waterproof pocket EC/TDS temp probe (Adwa Instruments, Szeged, Hungary), according to the method described by Valentovic *et al.* (2006). The TVD was cut into 1 cm^2 length discs and the weight recorded. The first electrolyte conductivity (EC1) reading was measured by placing the disc in a well of a 12-well

microtiter plate containing 2 ml deionized water at room temperature for 24 h. The discs were then frozen in liquid nitrogen, placed back into deionized water and disintegrated by vortexing and the second electrolyte conductivity (EC2) reading was recorded. EL was calculated as a percentage using the formula:

EL % = [(EC1)/ (EC2)] X 100

2.2.9.6 Quantifying malondialdehyde (MDA)

Hundred mg of powdered frozen plant tissue was homogenized in 500 µl of 6% (w/v) trichloroacetic acid (TCA) and centrifuged for 20 min at 5 000 g at 4^{o}C. The supernatant was collected and transferred to new sterile tubes and used for measurements of MDA according to the method of Heath and Packer (1968). Supernatant (200 µl) was added to the mixture of 400 µl of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was boiled at 90^{o}C for 20 min, cooled on ice for 10 min and centrifuged at 5 000 g for 5 min. Using a VesrsaMax ELISA Microplate reader, the absorbance was measured at a wavelength of 532 nm and 600 nm. MDA concentrations were calculated using the extinction coefficient of 155 mM.cm^{-1} and the following formula:

MDA (nmol)/g FW = ($\Delta A_{corrected}$*3*X*1000) / (Ɛ*b*y), where:

- $\Delta A_{corrected}$ = A532 - A600 corrected with ΔA of the blank
- b = light path length (0.631 cm for 200 µl)
- Ɛ = millimolar extinction coefficient (155 mM.cm^{-1})
- 3 = (dilution factor from 500 µl 6%TCA extract + 400 µl 20% TCA/0.5% TBA)
- X = (ml) TCA 6% used for extraction
- y = (g) FW used for extraction
- 1000 = conversion factor (mmol to nmol)

2.2.9.7 Reactive oxygen species (ROS)

In-situ detection of hydrogen peroxide (H_2O_2) was done according to the methods described by Thordal-Christensen *et al.* (1997) and Daudi *et al.* (2012) with minor modifications using

3, 3-diaminobenzidine (DAB). Superoxide radicals (O_2-) were detected using nitro-blue tetrazolium (NBT) staining according to a method described by Kumar *et al.* (2014). The TVD of transgenic sugarcane lines and wild-type sugarcane was cut into 1 cm length discs and placed in 2 ml of a 1 mg/ml DAB solution (200 mM Na_2HPO_4 and 0.05% (v/v) TWEEN 20; pH 3.8) or 3 mg/ml NBT dissolved in 10 mM potassium phosphate buffer (pH 7.8), and 10 mM NaN_3 for 30 min under vacuum. Discs were incubated on a shaker at room temperature for a further 24 h. Stained leaves were boiled at 70^{o}C for 1 h in ethanol: glacial acetic acid: glycerol (3:1:1 v/v) solution. Chlorophyll was removed by de-staining the discs in 96% ethanol at 60^{o}C for both DAB and NBT histochemical staining experiments and photographs were taken. In addition, according to Junglee *et al*. (2014), H_2O_2 was quantified by homogenizing 100 mg of ground frozen plant tissue in 500 µl of 0.1% (w/v) TCA and centrifuged (5 000 g, 15 min at 4^{o}C). Whilst working in the dark, the supernatant was collected and mixed in 1:2 ratio with 5 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide (KI). The mixture was placed in the dark at 4^{o}C for 1 h. The absorbance was measured using a VersaMax ELISA Microplate reader at 390 nm and H_2O_2 content was determined based on a standard curve which ranged from 0 – 2.5 mM of 12% (m/v) H_2O_2 (Supplementary data, Figure S1) and expressed as µM.mg^{-1}FW.

2.2.9.8 Catalase (CAT) and superoxide dismutase (SOD) measurements

For the estimation of antioxidant activity, 100 mg of ground frozen plant tissue was homogenized in 500 µl extraction buffer consisting of 0.1 M K_2HPO_4, 0.1 mM EDTA, and 1% (w/v) PVP at pH 7. The homogenate was vortexed and centrifuged at 5 000 g for 15 min at 4^{o}C. The supernatant was collected and quantified using the Bradford Assay (Bradford, 1976) (Supplementary data, Figure S2) and stored at -20^{o}C as template for catalase and SOD measurements. All antioxidant assays were conducted using three biological repeats of transgenic and wild-type sugarcane plants and readings were measured in triplicate.

Catalase activity was determined using the Catalase Kit (Sigma-Aldrich, Saint Louis, USA), according to the manual specifications. A standard linear range activity curve (Supplementary data, Figure S3) was prepared from reagents supplied in the kit and

measurements were done by quantifying the quinoneimine dye in a VersaMax ELISA Microplate reader at a wavelength of 520 nm. Catalase activity was expressed as nmol^{-1}min.mg^{-1}FW.

Superoxide dismutase activity was determined by using the SOD Assay Kit (Sigma-Aldrich, Saint Louis, USA), according to the manufacturer's guidelines. A standard activity curve was prepared using SOD (Cat number S8160, Sigma-Aldrich, Saint Louis, USA) at a concentration gradient of 0 to 20 units/ml (Supplementary data, Figure S4). The optical density (OD) was measured on a VersaMax ELISA Microplate reader at a wavelength of 450 nm and SOD was expressed as the rate at which it inhibits the oxidation of water-soluble tetrazolium salt (WST) from forming formazan.

2.2.9.9 Glutathione

Glutathione activity was measured according to a method described by Sahoo *et al*. (2017), which measures total, oxidized glutathione (GSSG) and reduced glutathione (GSH). For GSH assay measurements, 200 mg of ground frozen plant tissue was homogenized in 500 µl of 6% (w/v) metaphosphoric acid containing 1 mM EDTA. Homogenate was centrifuged (8 000 g, 15 min at 4^{o}C) and the supernatant (200 µl) was collected and 500 µl of 0.5 M potassium phosphate buffer (pH 7.5) added, the mix was kept on ice. The enzyme reaction was started by the addition of 100 µl each of 10 mM 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB), 10 mM bovine serum albumin (BSA) and 0.5 mM nicotinamide adenine dinucleotide (NADH). A standard curve was prepared by replacing extract with 0 - 50 µM GSH (Supplementary data, Figure S5). The reaction mix was incubated at 37^{o}C for 15 min, once cooled, reduced glutathione was measured using a VersaMax ELISA Microplate reader at a wavelength 412 nm and expressed as mM.mg^{-1}FW.

For the GSSG assay, 4 µl of 2-vinylpyridine was added to 200 µl supernatant of GSH extract and incubated at room temperature for 1 h. Hundred µl of this mix was added to 600 µl reaction buffer (100 mM potassium phosphate buffer, 5 mM EDTA; pH 7.5), 100 µl of glutathione reductase (GR, 20 U/ml) and 100 µl 10 mM 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction was started with the addition of 30 µl 2.5 mM nicotinamide adenine dinucleotide phosphate (NADPH) in 170 µl of the reaction mixture. Oxidized glutathione was

measured at a wavelength 412 nm for 5 min and 15 sec intervals. Total oxidized glutathione was then expressed as mM.mg^{-1}FW.

2.2.9.10 Proline

According to the method described by Bates *et al*. (1973), 50 mg of ground frozen plant tissue was homogenized in 250 µl of 3% (w/v) sulfosalicyclic acid and centrifuged (8 000 g, 15 min at 4^{o}C). One hundred µl of supernatant was added to a 500 µl reaction mixture containing 3% (w/v) sulfosalicyclic acid, 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M orthophosphoric acid. The mixture was boiled at 95^{o}C for 1 h. Once cooled, the absorbance of the reaction was measured at a wavelength 520 nm using a VesrsaMax ELISA Microplate reader. Proline concentration was determined using a standard activity curve which ranged from 0 - 100 mM proline (Supplementary data, Figure S6) and was expressed as µM.mg^{-1}FW.

2.2.9.11 Abscisic acid (ABA)

Abscisic acid was extracted using the method of Liu *et al*. (2014c) with minor modifications. Leaf tissue (400 mg) was lyophilized on a Virtis Bench Top freeze dryer (United Scientific, Cape Town, SA) for 24 h to obtain dry material. The dried material was homogenized in 500 µl chilled 80% (v/v) methanol on a shaker for 24 h in the dark at 4^{o}C. Homogenate was then centrifuged at 8 000 g for 10 min at 4^{o}C. The supernatant was lyophilized for 2 h. The dry pellet was re-suspended in 200 µl 0.1 M potassium phosphate buffer (pH 7) and vortexed for 30 sec. One hundred µl was used to quantitatively ABA content using the Phytodetek ABA Test Kit (Agdia, Indiana, USA) according to the manufacture's specification. Abscisic acid was expressed as nM.mg^{-1}DW.

2.2.10 Protein subcellular localization

2.2.10.1 Plant expression vector

A full-length *AtBBX29* amplicon was generated through PCR containing *Nco*I sites using the BBXNcoI Forw and Rev primer pair (Table 2.1). Primers were designed using Online In-Fusion

Cloning Tool software hosted by Clontech (https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-tools) and consisted out of two sections. The 5' end contained 15 bp that was homologous to the linearized vector, while the 3' end had sequence specific to the target gene. The pCambia1302 vector was linearized overnight with the *Nco*I restriction enzyme (NEB, Cutsmart buffer, 37°C). The amplicon was ligated into the *Nco*I site of the pCambia1302 binary vector containing the *GFP* reporter gene and 35S promoter, using the In-Fusion Cloning kit (Takara, USA), following the manufacturer's instruction. In pCambia1302:*AtBBX29*, the transgene was cloned in frame upstream of the reporter gene (Supplementary data, Figure S7). The recombinant vector was transformed using the standard heat-shock protocol into *E. coli* cells and selected on LB medium containing 50 mg/ml kanamycin. Colonies were screened by PCR using GoTaq and a combination of *GFP* gene specific and vector primers to confirm gene insertion (Table 2.1), followed by sequencing to confirm transgene integrity.

2.2.10.2 Agrobacterium tumefaciens transformation

Electro-competent *A. tumefaciens strain* LBA4404 cells were made according to section 2.2.3.3. One µl of pCambia1302:*AtBBX29* (100 ng/µl) and control pCambia1302 vector were individually electroporated into 100 µl of cell solution using the Gene Pulser Xcell electroporation system (BioRad Laboratories, USA) at 2500 V, 25 µF and 200 Ω in a 2 mm gap cuvette. *Agrobacterium* cells were mixed with 1 ml liquid LB medium (without antibiotics), agitated on a shaker at 225 rpm for 3 h at 28°C and selected on YEP medium containing 50 mg/ml kanamycin and 50 mg/ml rifampicin, following incubation at 28°C for 2 days. Putative transformed *Agrobacterium* cells were screened by PCR using GoTaq and a combination of *GFP* gene specific and vector primers (Table 2.1). Transformed *Agrobacterium* cells containing the rDNA were mix with 80% (v/v) glycerol and flash-frozen in liquid nitrogen and stored at -80°C for future use.

2.2.10.3 Agrobacterium transformation of onion epidermis cells

According to a method described by Xu *et al*. (2014), transformed *Agrobacterium* cells were cultured in 50 ml liquid YEP medium containing the appropriate antibiotics at 28°C until the

culture reached and OD_{600} of 1.5. Cells were harvested through centrifugation (5 000 g, 10 min at 4°C) and cell pellets were re-suspended in 25 ml infiltration liquid (41.65 mM D-glucose, 100 mM $CaCl_2$, 100 mM MES-KOH; pH 5.6, 0.011 µM BAP, 0.01% Silwet L-77[48], 50 mM $MgCl_2$ and 12.5 mM acetosyringone). *Agrobacterium* cells were then diluted in infiltration liquid until an OD_{600} of 0.1 was reached.

Locally cultivated red onions with medium to large bulbs were used as an experimental plant material. The onions were placed in the dark for 48-72 hours at 28°C. The tunic bulbs of the onions were removed and the outermost scales were cut into 4 sections allowing them to be refolded. Two hundred µl of the prepared *Agrobacterium* solution was inoculated between the interface of the adexial epidermis and the mesophyll using a narrow-gauge needle and syringe. The onion scales were refolded into their original positions and were held in place with adhesive tape. Onions were then covered with foil and co-cultivated in the dark for 72 h at 28°C. After co-cultivation, the region around the inoculation site, approximately 20 mm^2 sections of adexial epidermal area, were peeled from the onion scales and placed onto a droplet of water on the surface of a standard microscope slide and covered with a coverslip.

Onion cells were visualized for GFP fluorescence using a confocal microscope linked (Zen 2012; Central Analytical Facility (CAF), Stellenbosch University) to Zeiss imaging software. The cells were subjected to laser excitation of 458-510 nm and light was emitted between 520-550 nm at 4 x and 10 x magnifications and bright green field images were captured.

2.3. RESULTS

2.3.1 Transgene cloning into the plant expression vector

Through PCR, a 600 bp amplicon corresponding to the *AtBBX29* gene was amplified from pJet1.2:*AtBBX29* vector provided by Prof SK Panda, India. The amplicon was isolated and cloned into the pUbi510 plant expression vector. PCR analysis confirmed that *E. coli* colonies, 2 and 3 were successfully transformed with the pUbi510:*AtBBX29* recombinant vector (Figure 2.1).

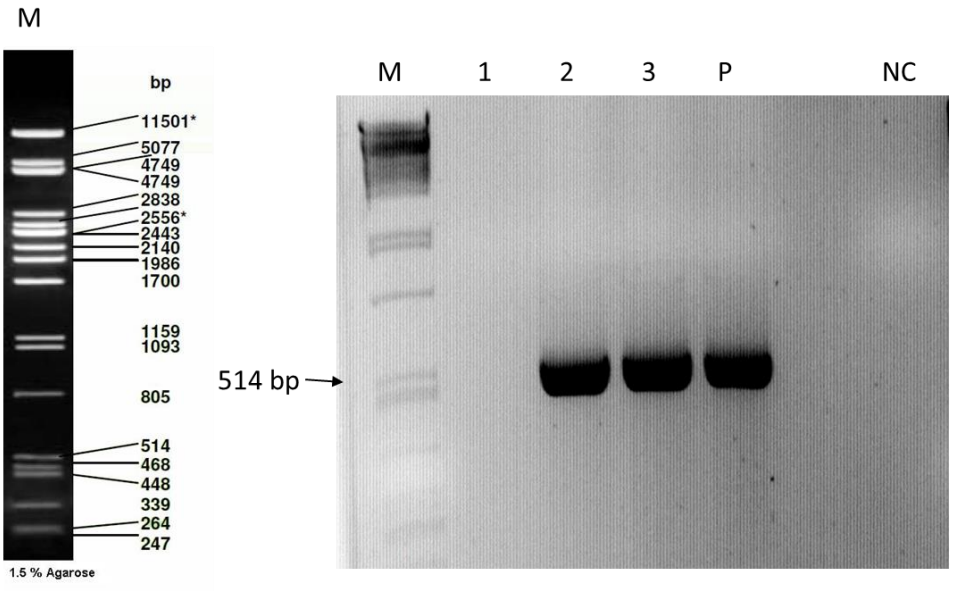

Figure 2.1. Colony PCR confirming cloning of the *AtBBX29* gene into the pUbi510 plant expression vector and transformation into the *E. coli* DH5α cells. Lanes M (*Pst*I λ marker); 1-3 (transformed *E. coli* colonies); P (positive plasmid control; pJet1.2:*AtBBX29*); (NC) (negative H_2O control).

2.3.2 Confirmation of sugarcane transformation

Sugarcane embryogenic calli were regenerated, bombarded with the pUbi510:*AtBBX29* vector and five putative transformed clones regenerated via somatic embryogenis (Figure 2.2).

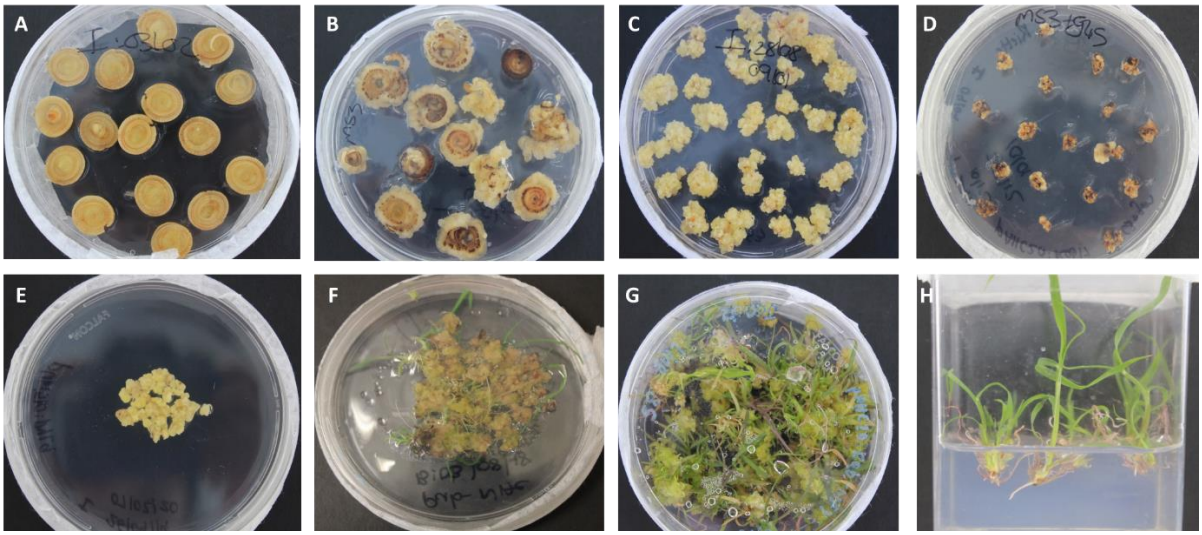

Figure 2.2. Generating *in vitro* putative transformed sugarcane (*Saccharum* spp. hybrid cv. NCo310) plantlets from bombarded embryogenic callus. Callus was initiated from **(a)** immature inner leaf roll explants isolated from stalk material; followed by the **(b)** development of callus and the harvesting of embryogenic callus **(c)** prior to transformation. **(d)** Bombarded callus was placed on selection media, and surviving calli **(e)** were allowed to develop somatic embryos **(f)**, which developed into sugarcane plantlets **(g)** that were allowed to elongate and form roots **(h)**.

Putative transformed sugarcane lines (T1.1, T1.6, T1.8, T2.1 and T2.9) were analysed for the presence of the *AtBBX29* transgene using PCR and gene specific primers. For this, genomic DNA (gDNA) was extracted from the leaves of putative transformed sugarcane and wild-type (WT) plants. An amplicon (600 bp) was successfully obtained from the genome of 4 transgenic lines (T1.1, T1.6, T1.8 and T2.1). No amplification was detected in the WT plant (Figure 2.3).

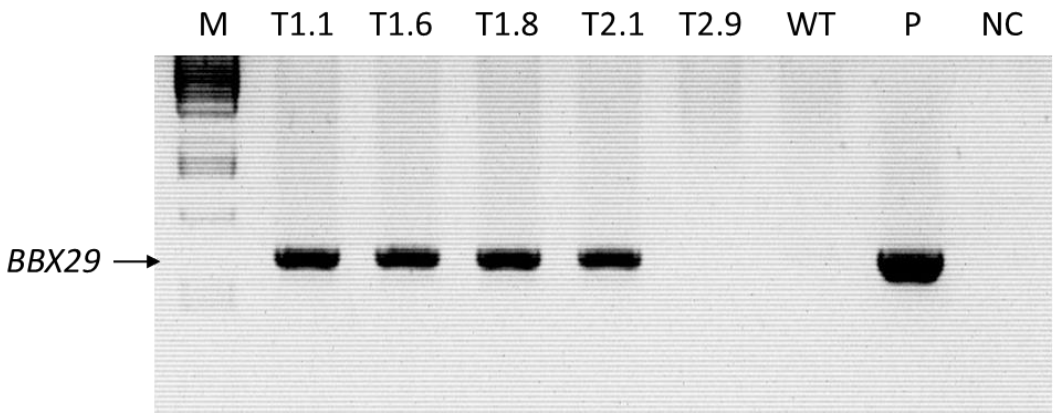

Figure 2.3. PCR analysis confirming the presence of the *AtBBX29* transgene in four of the putative transformed sugarcane lines (T1.1, T1.6, T1.8, and T2.1). M (*Pst*I λ marker); P (positive plasmid control; pJet1.2:*BBX29*); NC (negative H_2O control).

2.3.3 Expression analysis of transgene in transgenic sugarcane

Transformed sugarcane lines containing the *AtBBX29* transgene were further analysed for the relative expression of the inserted gene through semi-quantitative reverse transcriptase PCR (sqRT-PCR). RNA was extracted (Figure 2.4) and cDNA was synthesized from 3 transgenic (T1.1, T1.6 and T1.8) and WT plants. Gene expression levels were assessed by amplifying a 600 bp fragment using PCR primers specific to the *AtBBX29* gene (Figure 2.5 b) and the *Actin* reference gene (Figure 2.5 a). Gene expression levels obtained from the analysis indicated that *AtBBX29* was successfully expressed in all 3 transgenic lines. Two transgenic lines, T1.1 and T1.6 showed relative low levels of transgene expression, while transgenic line T1.8 relative high levels of *AtBBX29* expression. Semi quantitative–RT PCR analysis of the transgene expression in T1.8 furthermore resulted in a seemily double amplicon, not seen in the other two transgenic lines. No expression was detected in the WT plants (Figure 2.5 b).

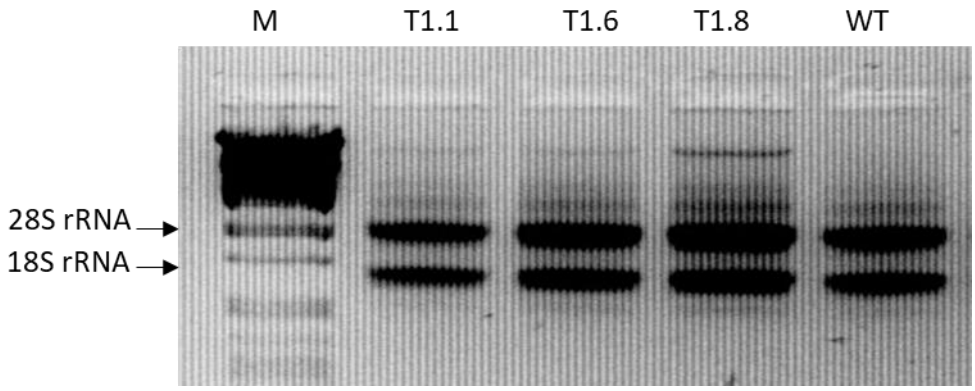

Figure 2.4 Gel electrophoresis of RNA extracts from the leaves of *AtBBX29* transformed sugarcane (lines T1.1, T1.6 and T1.8,) and WT plants using a Maxwell® 16 LEV Plant RNA Kit. M (*Pst*I λ marker). RNA separation was conducted on a 1% (w/v) TBE agarose gel, run at 100 V.

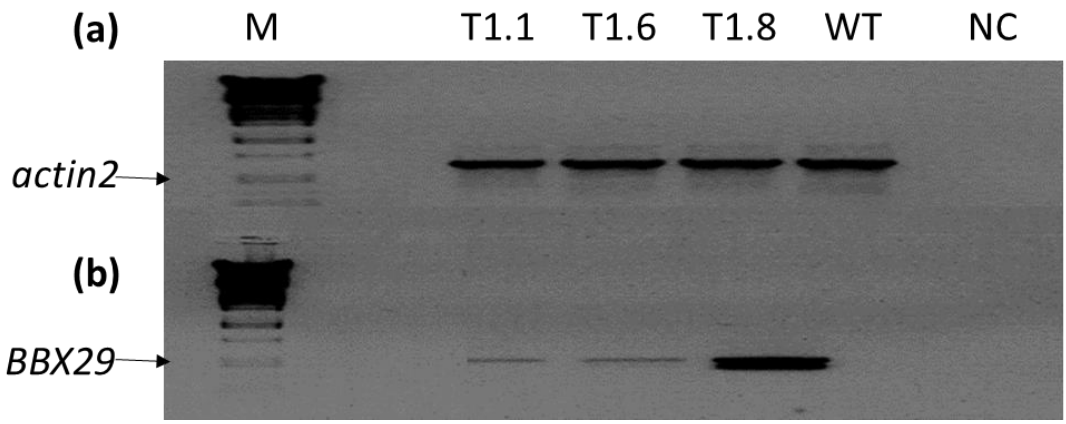

Figure 2.5. Semi-quantitative reverse transcriptase PCR (sqRT-PCR) showing expression levels of the *AtBBX29* transgene in transgenic sugarcane lines (T1.1, T1.6 and T1.8) and WT plants using **(a)** *Actin* primers as an internal control in comparison with **(b)** gene specific primers. M (*Pst*I λ marker); NC (negative H_2O control).

2.3.4 Agronomic traits of transgenic and WT plants under non-stressed environmental conditions

In order to assess the growth responses of untransformed and transformed plants containing *AtBBX29*, the plants were grown in a growth tunnel and phenotypically assessed after 8 months of growth under normal conditions with a consistent watering regime (Figure 2.6).

Figure 2.6. Maturing *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants in the growth tunnel on Welgevallen Experimental farm, Stellenbosch.

Agronomic parameters specifically associated with sugarcane yield and development towards the end of the grand growth phase were evaluated and compared to WT plants. Transgenic plants had no significant difference in internodes length and plant height compared to WT plants (Figure 2.7 c and d). T1.8 and T1.1 displayed significant differences in leaf length and width respectively, compared to the WT (Figure 2.7a and b).

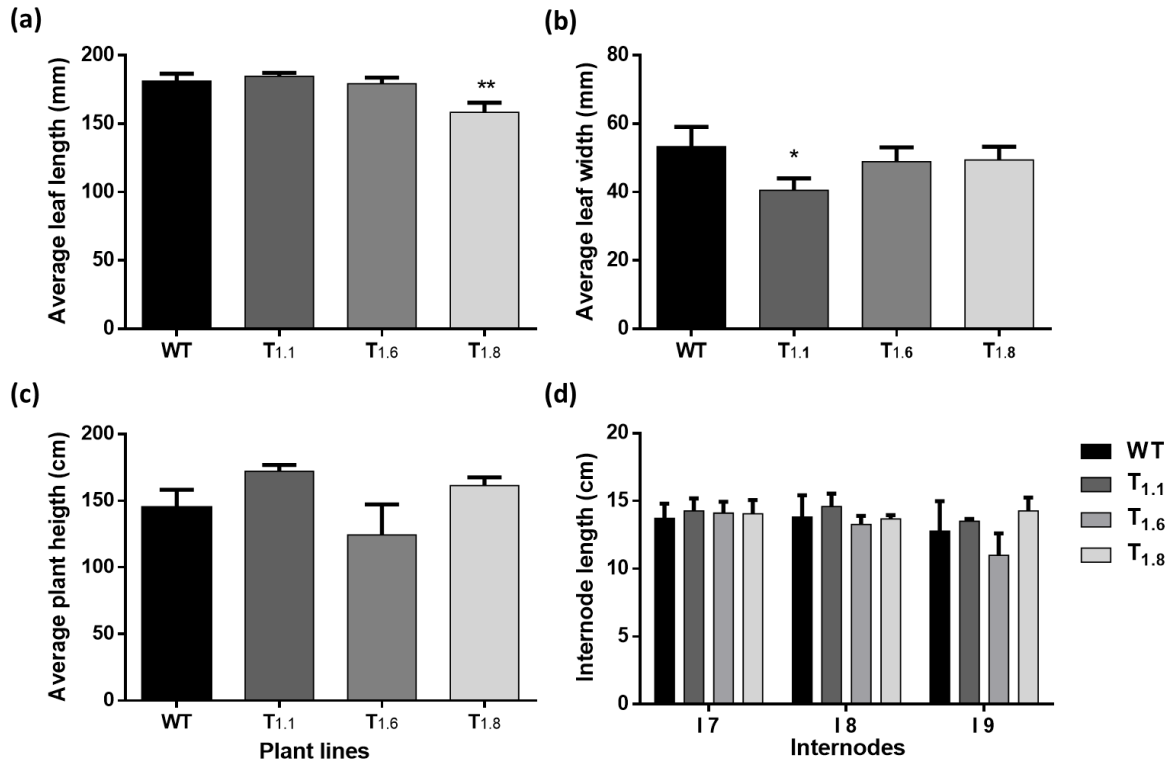

Figure 2.7. Phenotypic growth of *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants measured after 8 months of growth under normal environmental conditions. **(a)** Average leaf length (mm), **(b)** average leaf width (mm), **(c)** average plant height (cm) and **(d)** internode length (cm) were measured. Data is presented as means ± SD of three biological replicates (n = 3). (*) and (**) shows significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test.

In addition, the carbohydrate content were analyzed in the immature (I 3) and mature (I 9) internodes of *AtBBX29* transgenic and WT plants. As expected, total sugars (which is defined by the contents of sucrose, glucose and fructose) were much higher in the mature versus immature internodes, at an average fold increase of 10. Most importantly, sucrose levels were not significantly different in the tissue types between the transgenic and WT plants (Figure 2.8 a). However, while glucose and fructose levels were similar in immature cane between the difference genotypes, variation and significant lower levels of glucose and fructose were seen in mature internodes in some of the transgenic lines (T1.1 and T1.8) compared to WT (Figure 2.8 b and c).

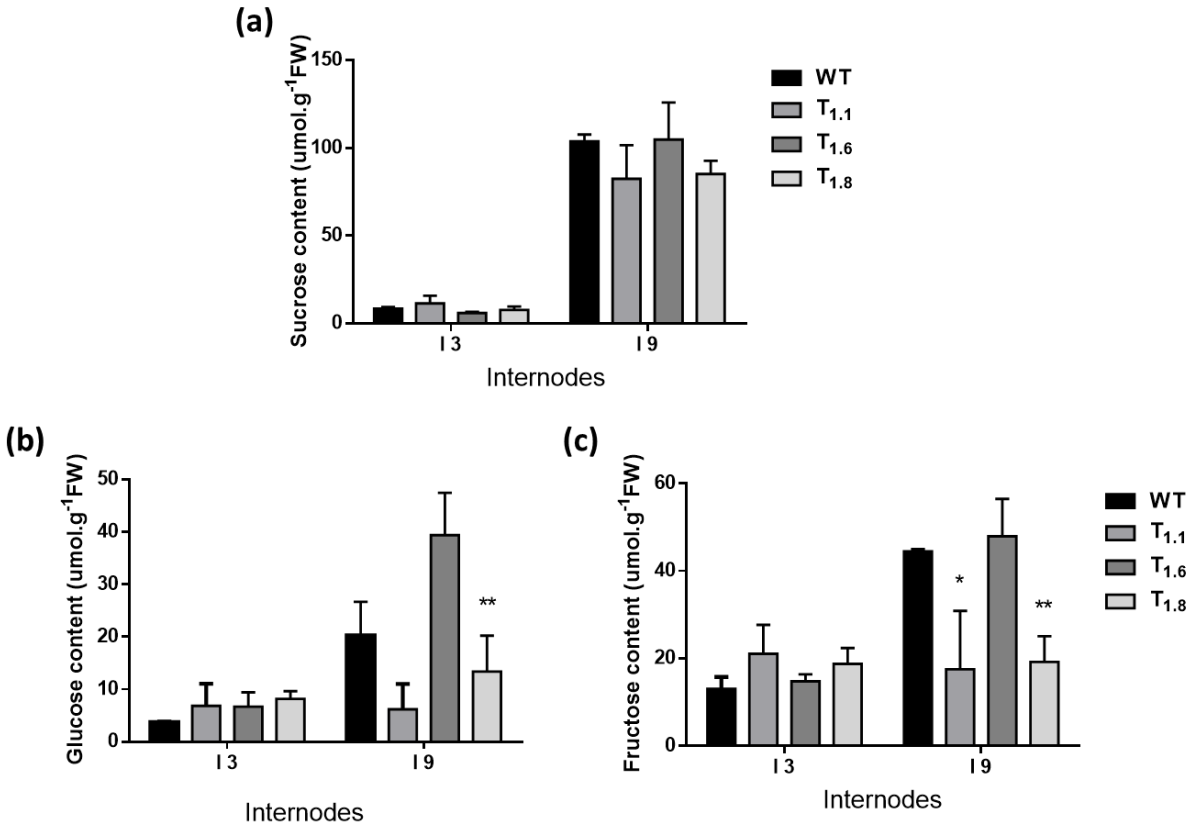

Figure 2.8. The levels of total soluble sugars in the immature and matured internodes (I 3 and I 9) of *AtBBX29* transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants measured after 8 months of growth under normal environmental conditions. Measurements include **(a)** sucrose; **(b)** glucose and; **(c)** fructose content. Data is presented as means ± SD of nine biological replicates (n = 9). (*) and (**) shows significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test.

2.3.5 Phenotypic response of WT and transgenic sugarcane exposed to drought

Four months old *AtBBX29* transgenic sugarcane, lines T1.1, T1.6 and T1.8, and WT plants were exposed to 21 days of water-deficit stress. These plants were monitored for their phenotypic response and survival rates during and after the drought period in comparison to WT plants. Ultimately the different sugarcane genotypes displayed no significant difference in growth and the non-stress phenotype, as observed at the start of the water

deficit stress period (day 0 ww), was mostly maintained under mild drought stress conditions (day 7 ww) (Figure 2.9 a and Table 2.3).

The WT started to exhibited stress symptoms by day 14 ww with 47% of the plants showing signs of chlorosis along with growth retardation, while leaf length dropped from an average 126 to 114 cm and plant height from 43 to 40 cm (Figure 2.9 a; Table 2.2). The WT plants also started to die with a confirmed 6% of the plants that were dead by day 14 ww, with leaves being completely dry and brittle. This in contrast to all the transgenic lines, which between 77-83% of the plants showing no signs of stress and only between 17-23% of plants showed minimal damage, such as yellowing of leaf tips (Figure 2.9; Table 2.3). At this stage transgenic plants also maintained growth with plant height increasing between 2-4 cm for the different genotypes (Table 2.3).

Extended drought to 21 days ww increased WT plants death (63%) whereas most transgenic lines T1.1 and T1.6 survived longer (between 40 and 50%) and remained healthy (Figure 2.9; Table 2.3). However, by 21 days ww transgenic line T1.8 showed severe signs of stress and the majority of the plants have died (73%). Throughout the 21 days of water-deficit stress, transgenic lines and WT plants leaf width remained between 3-4 cm. No significant difference was observed in the response of leaf length and plant height between transgenic and WT plants. However, by day 21 ww leaf length was reduced to 114 cm in the WT plants, an average decrease of 12 cm, while the transgenic lines remained mostly unaffected. The same response was observed in the values obtained for plant height where transgenic plants tended to be higher than WT plants but not at significant levels due to increase variations seen between the individual plants within a genotype.

Plants were watered 2 days post the 21 days water-deficit stress period. Twenty percent of transgenic plants recovered successfully with the development of new green leaves and normal growth resumed. However, only two WT plants, thus 4% of plants included in the trial, was able to recover and showed signs of renewed growth (Figure 2.9 b).

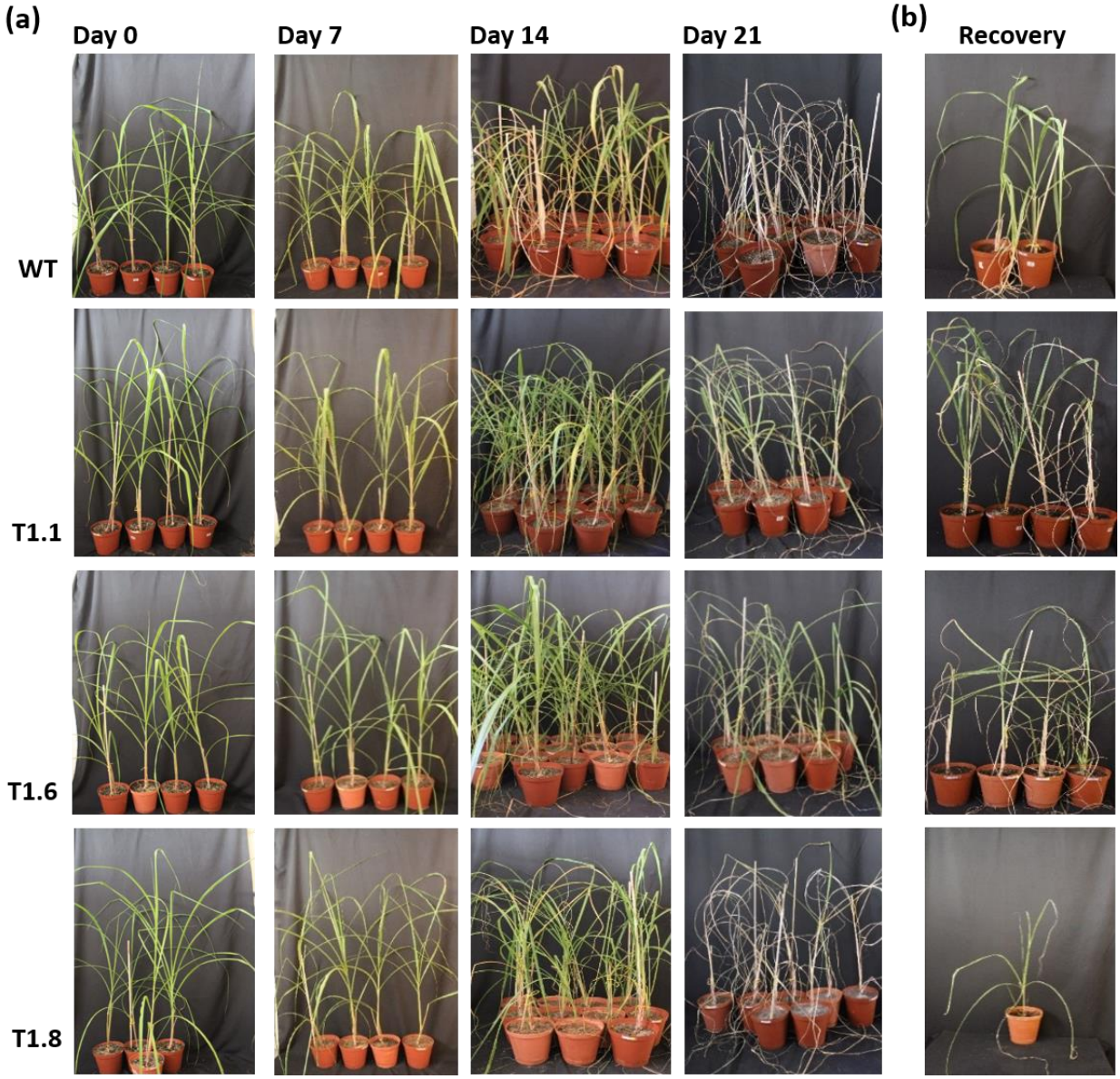

Figure 2.9. Phenotypic variation of *AtBBX29* transgenic sugarcane lines (T1.1, T1.6 and T1.8) and wild-type (WT) plants under drought. Drought was initiated by deprivation of water for a period of 21 days**.** Phenotypic analysis was evaluated every 7 days of water deprivation**. (a)** Phenotypic changes at days 0, 7, 14 and 21; and **(b)** recovery after re-watering for 14 days.

Table 2.2 Leaf width, length and shoot height of *AtBBX29* transgenic and WT plants evaluated every 7 days for the duration of the 21 day water-deficit period. Values present the average means ± standard deviation (SD) of four biological replicates (n = 4). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

Plant line	Average leaf width (cm)	Average leaf length (cm)	Average plant height (cm)
Day 0			
WT	4 ± 0.3	126 ± 7	43 ± 4
T1.1	3 ± 0.4	123 ± 6	46 ± 6
T1.6	4 ± 0.3	119 ± 13	45 ± 4
T1.8	3 ± 0.3*	130 ± 13	48 ± 3
Day 7			
WT	4 ± 0.3	128 ± 8	45 ± 3
T1.1	3 ± 0.4	127 ± 4	50 ± 6
T1.6	4 ± 0.3	119 ± 7	47 ± 5
T1.8	3 ± 0.5*	134 ± 10	49 ± 3
Day 14			
WT	4 ± 1	114 ± 16	40 ± 7
T1.1	3 ± 0.7	117 ± 5	50 ± 6
T1.6	4 ± 0.5	119 ± 8	48 ± 5
T1.8	3 ± 0.6	122 ± 12	50 ± 3
Day 21			
WT	4 ± 0.7	114 ± 15	40 ± 7
T1.1	3 ± 0.6	120 ± 7	48 ± 2
T1.6	4 ± 0	119 ± 6	48 ± 6
T1.8	3 ± 0.6	122 ± 10	50 ± 3

Table 2.3 Survival rates (%) of *AtBBX29* transgenic sugarcane and WT plants exposed to 21 days of drought. For each genotype a total of 25 plants were included in the pot trial. Plants were recorded as healthy when less than 5% of the leaves showed signs of browning. Values represent the number of plants being dead, damaged or healthy at each time point, expressed as a percentage.

Plant line	Dead (%)	Damaged (%)	Healthy (%)
Day 14			
WT	6	47	47
T1.1	0	21	79
T1.6	0	17	83
T1.8	0	23	77
Day 19			
WT	38	24	38
T1.1	39	17	44
T1.6	20	27	53
T1.8	40	18	42
Day 21			
WT	63		37
T1.1	60		40
T1.6	50		50
T1.8	73		27

The levels of water loss experienced by the different genotypes and the relative water content (RWC) of leaves, which in turn coincides with the analysis of soil moisture content (SMC), were also assessed (Figure 2.10). A saturation of water in the soil (± 0.4 m^3/m^3) and leaves of transgenic lines and WT plants were obtained prior to the induction of water-deficit stress (day 0 ww). Thereafter, the soil moisture content of all pots included in the trial gradually decreased at almost similar rates to nearly zero (± 0.025 m^3/m^3) by day 21 ww (Figure 2.10 a). The SMC of the pots containing WT plants did tend to drop quicker between days 0 to 14 ww but it was not significantly different when compared with pots containing transgenic plants. Over the course of the drought stress period all plants lost between 35-40% of their RWC (Figure 2.10 b). However, the RWC in the transgenic *AtBBX29* plants did not differ significantly from the non-transformed plants. Only transgenic line T1.8 under severe water stress (day 21 ww) showed significantly lower levels of RWC when compared with the WT.

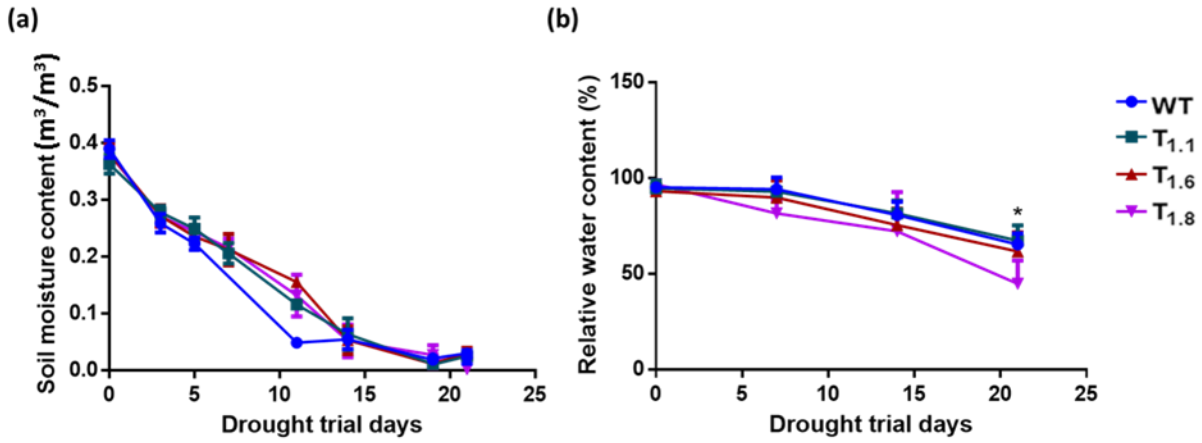

Figure 2.10. Comparative analysis of **(a)** soil moisture content of all pots and **(b)** relative water content (%) in the leaves of transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants prior to and after induction of water-deficit stress. Data is presented as means ± SD of four biological replicates (n = 4). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

2.3.6 Lipid peroxidation and membrane damage in transgenic sugarcane exposed to drought

Oxidative damage was estimated by measuring the final products of lipids peroxidation in transgenic and non-transformed plants exposed to water-deficit stress. In addition, the electrolyte leakage (EL; %) was measured to reflect the potential cell membrane damage. MDA content remained low and at equal levels in transgenic and WT plants during the early days of water-deficit stress (7 days ww) (Figure 2.11 a). By day 14 ww the MDA content increase significantly in transgenic lines T1.1 and T1.6, when compared to the WT plants. However, under severe stress (21 days ww) the MDA content in the WT plants was significantly higher than the levels in most transgenic lines, with only T1.8 not being significantly lower than the WT. The EL levels stayed the same in all the genotypes across the water-deficit stress period (Figure 2.11 b).

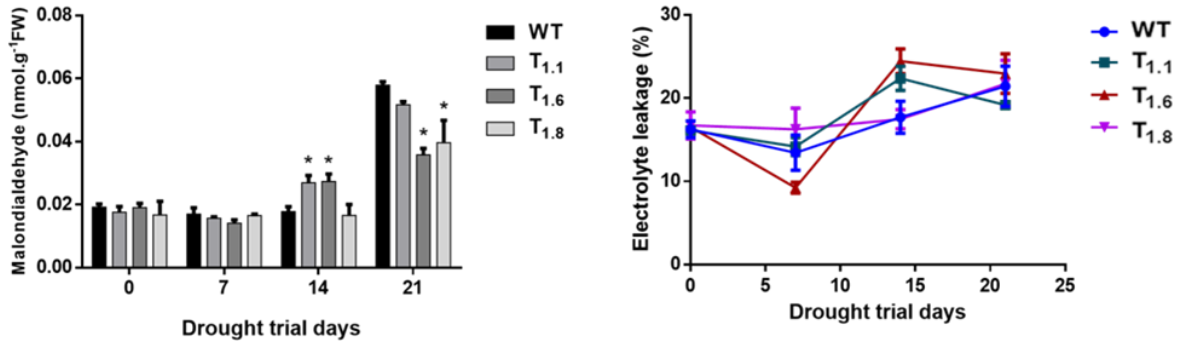

Figure 2.11. Estimation of cellular damage in transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants under drought stress. Measurement of average **(a)** malondialdehyde (MDA) content and; **(b)** electrolyte leakage. Error bars designates standard deviation (SD) of the means of three biological replicates (n = 3). (*) shows significant differences compared to the WT at $p \leq 0.05$. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

2.3.7 Oxidative stress in transgenic and WT sugarcane under drought stress

Reactive oxygen species (ROS), known to increase under stress conditions, were detected qualitatively by DAB and NBT staining for the production of hydrogen peroxide and superoxide, respectively (Figure 2.12). All lines, WT and transgenic, showed low production of ROS indicated by a weak staining of both DAB and NBT, under non-stressed conditions (day 0 ww). As the drought stress was extended (days 7, 14 and 21 days ww), some increase in dark brown and blue staining streaks, especially outside the midrib area of the leaf structure, were observed in the WT plants and to a lesser extend in the transgenic plants. Especially accumulation of O_2^- was more prominent in the leaves of WT plants than transgenic lines (Figure 2.12 a). However, because staining patterns was not easy to visually assess, the accumulation levels of H_2O_2 were further confirmed by quantitative analysis (Figure 2.13). On day 7 and 14 ww, the increase in the levels of H_2O_2 was significantly lower in all transgenic lines compared to the WT plants. Under severe stress, 21 days ww, WT plants accumulated more H_2O_2 while transgenic lines T1.1 and T1.6 had significantly reduced ($p \leq 0.01$) levels (Figure 2.12 b).

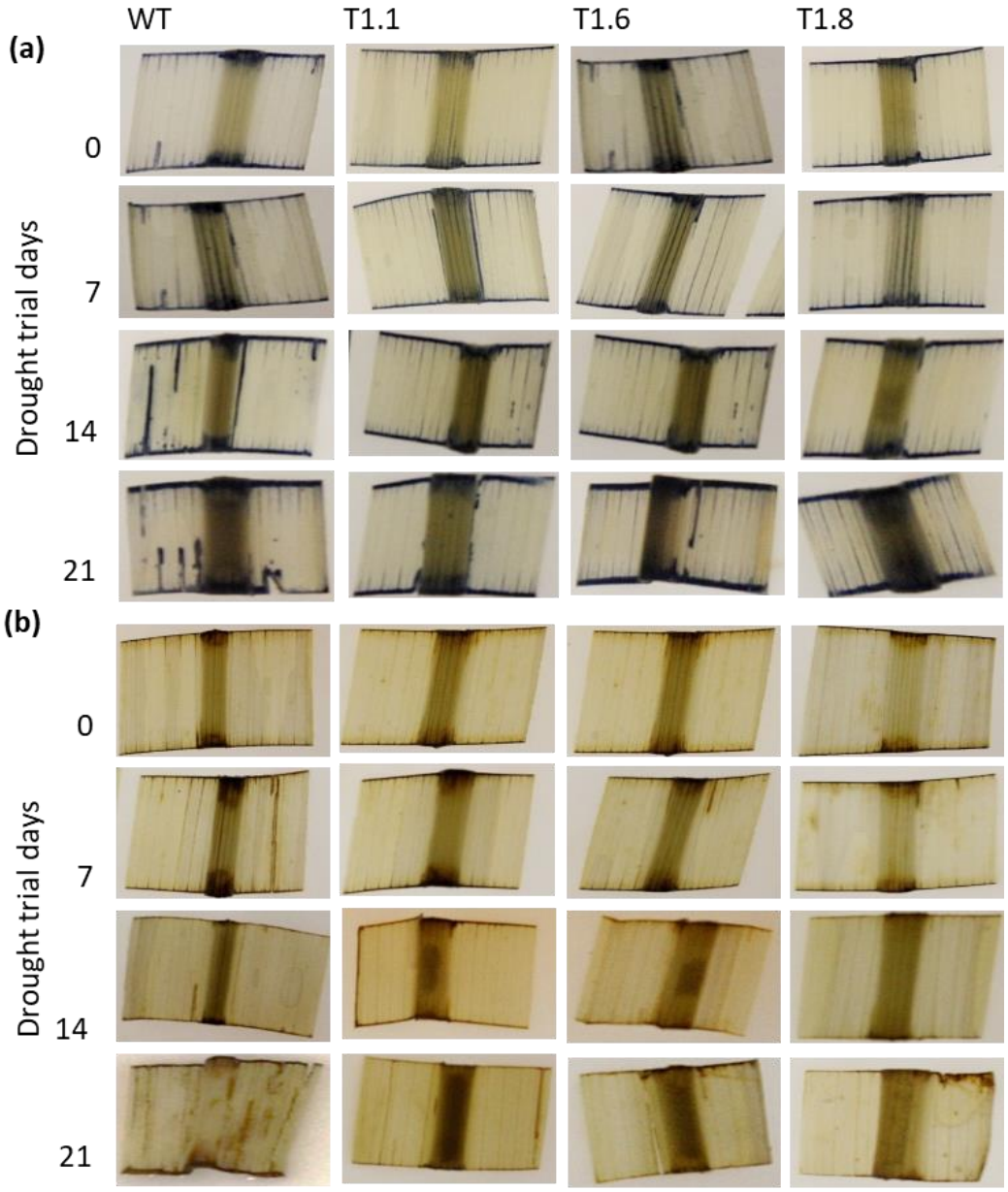

Figure 2.12. Histochemical analysis of reactive oxygen species (ROS) accumulation in the leaves of transgenic (T1.1, T1.6 and T1.8) and WT plants under drought stress, on days 0 to 21 without water. **(a)** Nitro-blue tetrazolium (NBT) staining of superoxide radicals (O_2^-) and; **(b)** 3,3-diaminobenzidine (DAB) staining of hydrogen peroxide (H_2O_2).

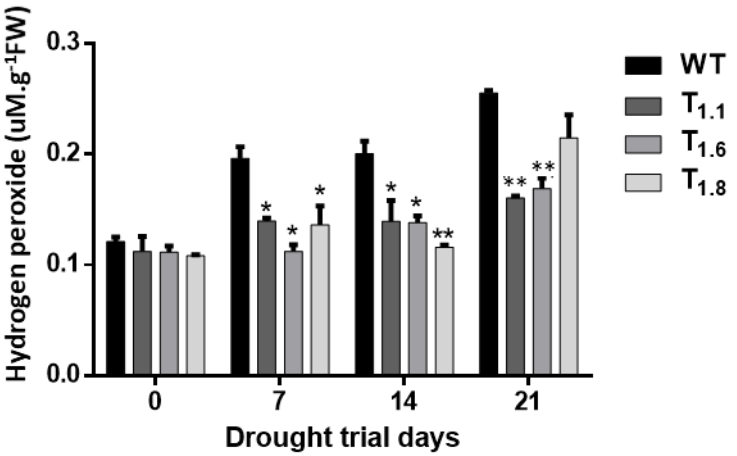

Figure 2.13. Quantitative analysis of hydrogen peroxide (H_2O_2) levels in transgenic (T1.1, T1.6 and T1.8) and WT plants under drought stress. Data is presented as means ± SD of three biological replicates (n = 3). (*), (**) shows significant difference compared to the WT at $p \leq 0.05$ and $p \leq 0.01$, respectively. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

2.3.8 Antioxidant response and compatible osmolyte accumulation in transgenic and WT plants under water-deficit stress

In defence against oxidative stress, plants generate antioxidants which comprises of enzymatic and non-enzymatic activities. The change in enzymatic antioxidants, superoxide dismutase (SOD) and catalase (CAT) were examined in transgenic and WT plants exposed to 21 days of water-deficit stress. Prior to drought stress (day 0 ww), SOD activity was similar among transgenic lines and WT plants and remained unaffected in all genotypes till drought was extended to 14 days ww (Figure 2.14 a). However, by day 21 ww the SOD content in transgenic lines T1.1 and T1.8 were significantly higher than those detected in WT. On the other hand, CAT activity increased steadily during the drought stress regime in all transgenic lines, with T1.8 being significantly higher than WT plants from mild (days 7 and 14 ww) till severe (day 21 ww) stress condition (Figure .2.14 b). By day 21 ww all transgenic lines had significantly higher ($p \leq 0.01$) CAT than the WT.

The non-enzymatic activity of reduced glutathione (GSH) and oxidized glutathione (GSSG) accumulation in transgenic and WT plants were investigated under water-deficit stress. Prior to the induction of water-deficit (day 0 ww) GSH levels were the same in all genotypes and increased in all plants as the drought stress extended (Figure 2.14 d). Nevertheless, there were no significant differences in GSH levels between the WT and transgenic lines. GSSG levels tended to be higher in the transgenic lines, with T1.8 levels being significantly higher, compared to the WT prior to drought (day 0 ww; Figure 2.14 d). However, by day 14 ww the GSSG content in the transgenic lines decreased, with transgenic line T1.8 being significantly lower than WT plants. By day 21 ww, the GSSG levels seem to be similar in all genotypes (Figure 2.14 d).

To study the level of osmolyte accumulation in the different types of plants, proline content was assessed (Figure 2.14 c). Initially WT and transgenic plants had similar low levels of proline (day 0 ww). These levels remained low under mild stress (day 7 ww) in all the genotypes but by day 14 ww, the proline levels increased significantly in transgenic lines T1.1 and T1.8 ($p \leq 0.001$ and $p \leq 0.01$) compared to the WT plants. Under severe stress (day 21 ww) all the transgenic plants had significantly higher levels of accumulated proline. These results indicate the possibility that the overexpression of *AtBBX29* led to osmotic adjustment in the transgenic plants.

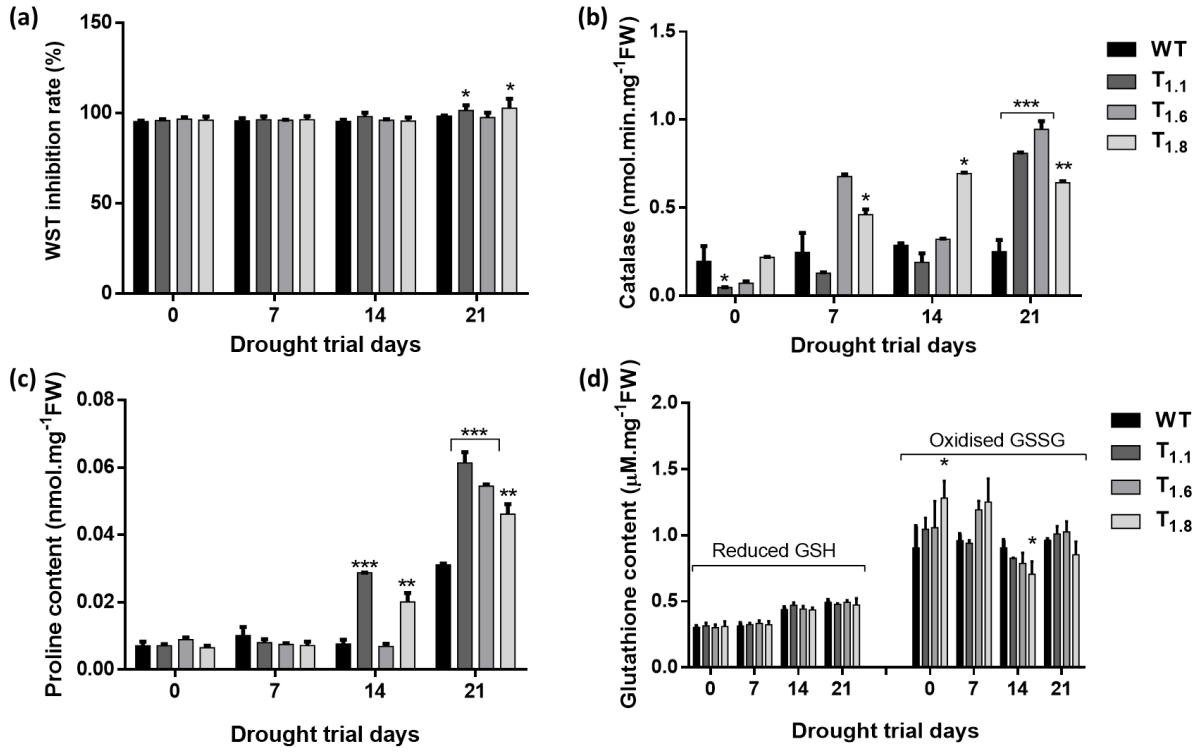

Figure 2.14 Estimation of antioxidant and osmolyte activity in transgenic (T1.1, T1.6 and T1.8) and WT plants exposed to water-deficit stress. Measurements include **(a)** superoxide dismutase (SOD) activity, presented as the rate of WST inhibition; **(b)** catalase; **(c)** proline content and; **(d)** glutathione content. Data is presented as means ± SD of three biological replicates (n = 3). (*), (**) and (***) shows significant differences compared to the WT values at $p \leq 0.05$, 0.01 and 0.001, respectively. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

2.3.9 Photosynthetic activity in transgenic sugarcane and WT under drought stress

The role of *AtBBX29* in the photosynthetic capacity of the plants during the 21 days of drought stress were investigated by measuring stomatal conductance, chlorophyll fluorescence (Fv/Fm) and chlorophyll content. Under non-stress conditions (day 0 ww), chlorophyll fluorescence (Fv/Fm) was similar in the different plant types and remained unchanged in the WT for approximately the next 12 days ww. However, transgenic line T1.1 showed significantly higher photosynthetic rates on days 5 and 11 ww compared to the WT plants (Figure 2.15 a). By day 14 ww, the photosynthetic rate varied greatly among transgenic lines and WT plants and the rate in transgenic line T1.8 decreased significantly

compared to the WT. By the end of the drought trial all transgenic lines maintained a high photosynthetic rate, significantly higher with T1.8 and T1.1, than WT plants.

The initial (± 90 mmol/m^2s) stomatal conductance was increased to ± 150 mmol/m^2s in WT and transgenic lines during the early days of drought (days 0 to 5 ww). After 10 days ww the stomatal conductance of WT plants decreased and all transgenic lines had significantly higher levels of conductance. Transgenic lines maintained these significantly higher levels of stomatal conductance, compared to the WT, throughout the rest of the drought stress period even under severe (day 21 ww) stress conditions (Figure 2.15 b).

The chlorophyll content was similar in the WT and transgenics at the onset of the induced water-deficit stress and declined as the drought period was extended. The transgenic lines remained slightly higher levels of chlorophyll under mild stress (day 7 and 14 ww). However, no significant difference was observed in the total chlorophyll content throughout drought stress periods between the different genotypes (Figure 2.15 c).

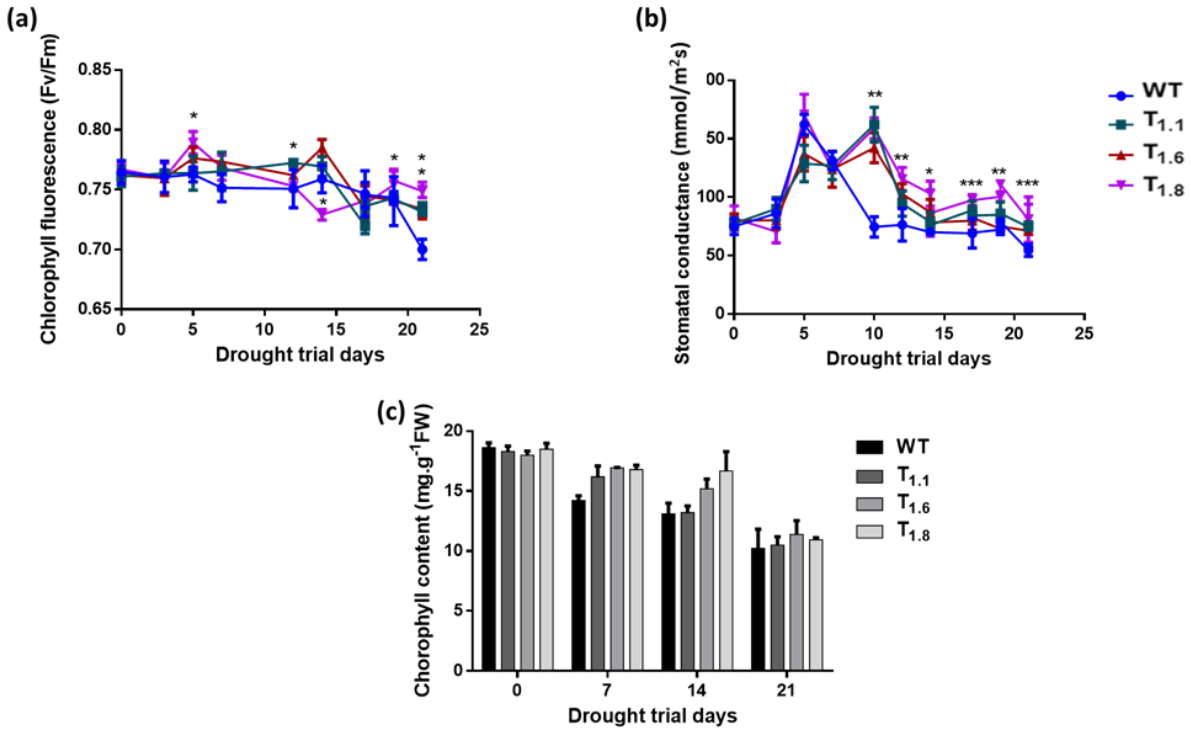

Figure 2.15. Analysis of photosynthetic machinery of transgenic sugarcane (T1.1, T1.6 and T1.8) and WT plants prior to (day 0) and after exposure to water-deficit stress (days 7, 14 and 21 ww). Measurements include the **(a)** chlorophyll fluorescence (Fv/Fm); **(b)** stomatal conductance and; **(c)** chlorophyll content of the different plants. Data is presented as means ± SD of four biological replicates (n = 4). (*), (**) and (***) indicate significant differences compared to the WT at $p \leq 0.05$, 0.01 and 0.001, respectively. Statistical significance was determined using one-way ANOVA and variance using Tukey's multiple comparison test.

2.3.10 ABA accumulation in transgenic and WT plants in response to drought stress

Endogenous abscisic acid (ABA) levels were measured in the leaves of transgenic (T1.1 and T1.8) and WT plants. No measurement could be done for T1.6 because of insufficient material. Results showed no significant difference in the content of ABA between transgenic and WT plants. Prior to stress induction (day 0 ww), the WT had higher ABA levels than the two transgenic lines though not significant. The initial ABA levels dropped under mild stress (days 7 and 14 ww) in all the different plant types. Under severe drought stress (day 21 ww), ABA content in T1.8 increased and was higher than that of WT plants but due to larger variation seen between individual plants in the test pool this was not significant (Figure 2.16).

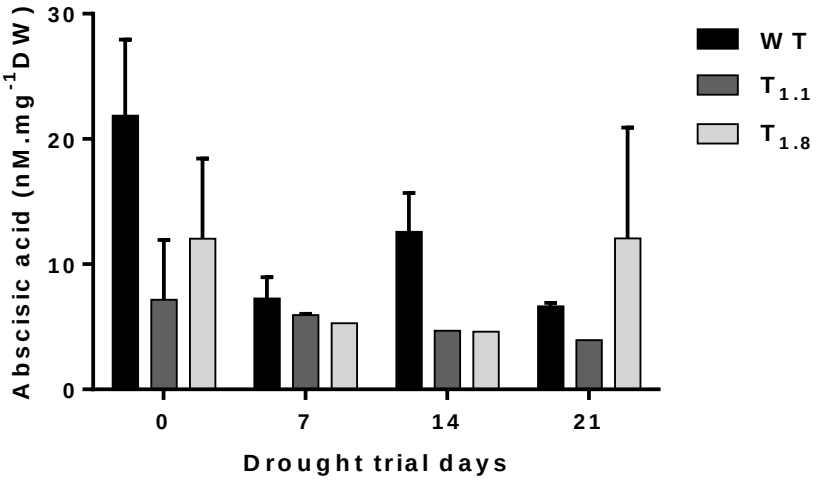

Figure 2.16. Abscisic acid (ABA) content in the leaves of *AtBBX29* transgenic sugarcane and WT plants exposed to water-deficit stress. Data is presented as means ± SD of three biological replicates (n = 3). Statistical significant differences between the genotypes at $p \leq 0.05$ was not observed.

2.3.11 Subcellular localization

2.3.11.1 Cloning and transformation of AtBBX29

For the *AtBBX29* localization studies, the binary pCambia1302 vector was used, containing the gene coding for the green fluorescent protein (*GFP*), and the full length *AtBBX29* sequence was cloned upstream of the *GFP* reporter (Supplementary data, Figure S1). The fusion gene cassette (*AtBBX29:GFP*) was then placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and *nopaline synthase (nos*) terminator. The full length *AtBBX29* gene was obtained through PCR using pJET1.2:*AtBBX29* as template and gene specific primers, which were designed to eliminate the gene stop codon and contain an additional *Nco*I site for cloning purposes (Figure 2.17). The obtained amplicon was cloned into a linearized pCambia1302 vector and the recombinant plasmid was then transformed into E. *coli* DH5α competent cells (Figure 2.18 a) and subsequently into *Agrobacterium tumefaciens* LBA4404 cells via electroporation (Figure 2.18 b). Successful cloning and transformation was confirmed by a 600 bp amplicon obtained from both bacterial cell clones.

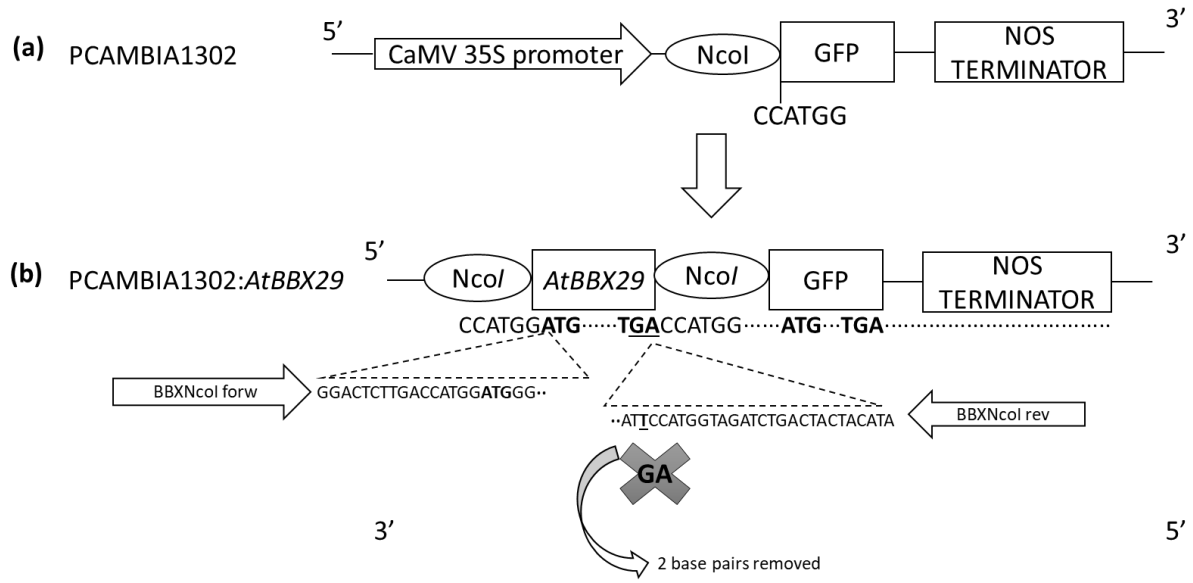

Figure 2.17. Diagram illustrating the fusion of the *AtBBX29* gene upstream of the *GFP* reporter gene in the pCambia1302 vector. BBXNcoI forw is a primer designed to carry the CCATGG sequence and a BBXNcoI rev primer designed to remove the stop codon (**TGA**: 2 bases removed) from the BBX29 sequence in frame with the start codon (**ATG**) of *GFP*.

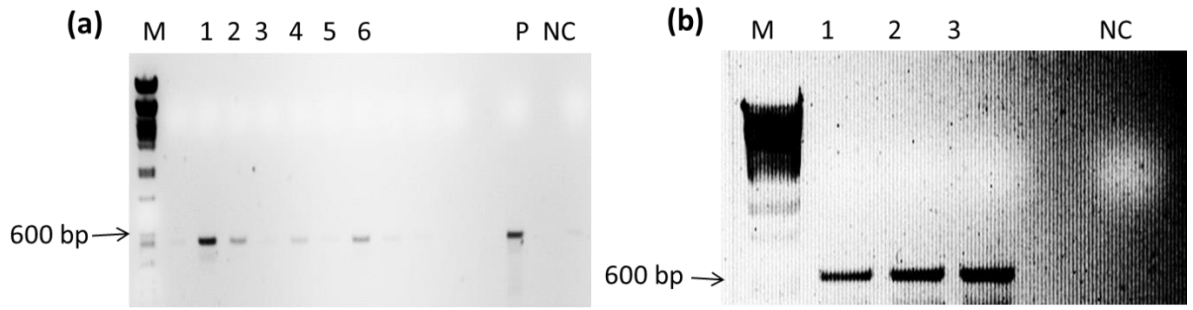

Figure 2.18. Colony PCR analysis confirming the cloning of the **(a)** full length *AtBBX29* gene (lanes 1-6) into the pCambia1302 plant expression vector and transformation into the *E. coli* DH5α cells; and **(b)** transformation of pCambia1302:*AtBBX29* into *Agrobacterium tumefaciens* (lanes 1-3). Lane M (*Pst*I λ marker); P (positive plasmid control; pJet1.2:*BBX29*); NC (negative H_2O control).

2.2.11.2 *Agrobacterium transformation and histology analysis of transient gene expression*

The pCambia1302:*AtBBX29* construct was transfected into onion epidermis cells via *Agrobacterium*-mediated infiltration, using an empty vector (pCambia1302) as control. The transiently expressed *AtBBX29:GFP* fusion was viewed 72 hours after incubation in the dark. Baseline fluorescence was present throughout the cells in both the control and recombinant vector construct (Figure 2.19), with only very faint green fluorescence spots seen in the microscope field. Upon further investigation, information on the pCambia website confirmed that the *GFP* sequence used in the initial pCambia vectors convey poor fluorescence in plant cells (https://cambia.org/welcome-to-cambialabs/cambialabs-projects/cambialabs-projects-legacy-pcambia-vectors-pcambia-legacy-vectors-1/cambialabs-projects-legacy-pcambia-vectors-list-of-legacy-pcambia-vectors-3/). Furthermore, due to limited capacity at the Central Analytic Facility-Microscope Unit, we were unable to do a time-based analysis of the transient expression profile to determine the best time, post infection, to detect the optimal transient fluorescent signal. This experiment will have to be repeated since no fluorescence was obtained that could to be associated with *GFP* and localization of the *AtBBX29* gene.

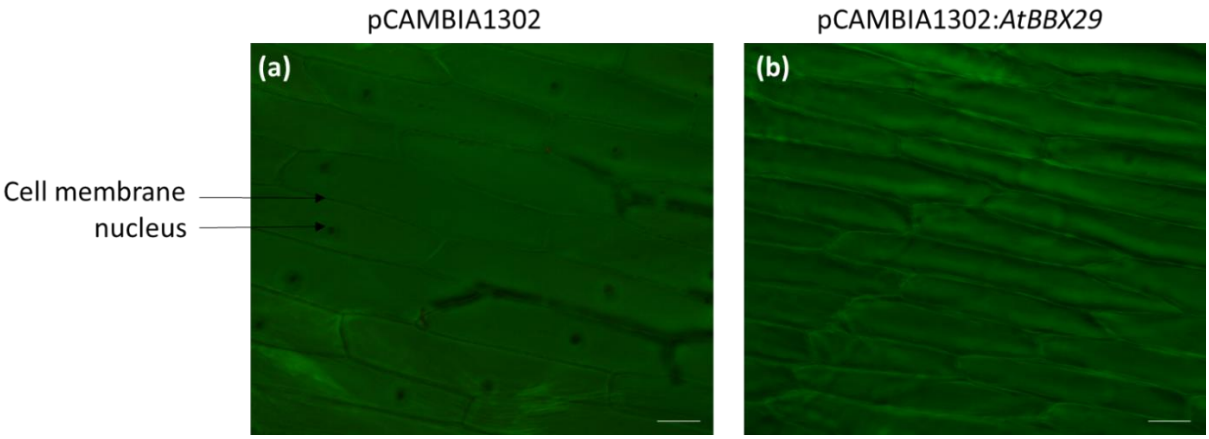

Figure 2.19. Histology analysis of the transient gene expression of *GFP* to determine the subcellular localization of the *AtBBX29* gene in onion epidermis cells transformed with the **(a)** pCambia1302 control vector or; **(b)** pCambia1302*:AtBBX29* recombinant vector construct. Bar = 10 µm

2.4 DISCUSSION

The responses of plants to an environmental stress is known to consist of altered gene expression and cellular metabolism leading to changes adaptive for growth, productivity and survival. Drought, taking in account the severity of this stress, can cause these physiolocial and biochemical changes (Anjum *et al.,* 2011; Shao *et al.,* 2009). In sugarcane, growth impairment is a reduction in both cell division and elongation (Inman-Bamber and Smith, 2005). Increase tolerance to water-deficit stress in sugarcane, achieved through transgenic research, has been reported only in a few cases and has been accomplished by targeting genes known to be responsive to water stress or known to provide water stress tolerance in other plant species (Augustine *et al.,* 2015; Ramiro *et al.,* 2016; Reis *et al.,* 2014).

Literature shows that the B-box zinc finger (BBX) proteins play essential roles in plant growth and development, and the function of BBX proteins in regulating flowering and light morphogenesis have been extensively studied (Khanna *et al*., 2009; Kumagai *et al*., 2008). However, the roles of BBX proteins in abiotic stresses are still poorly understood. Overexpression of the genes coding for these proteins in transgenic plants have been linked either positively or negatively to plant developmental processes and essentially regulate a network of stress responsive genes for plant adaptation (Zhang *et al.,* 2016). To date in the *Poaceae* species, the *OsBBX1*, *OsBBX2*, *OsBBX8*, *OsBBX19* and *OsBB24* genes have been linked to abiotic stress responses, including drought, through changes in their expression profiles, which potentially signifies roles in the plant's stress reponse (Shalmani *et al*., 2019). In addition, a few BBX genes have been functionally annotated in regards to drought stress. Overexpression of *ZFP* genes, namely *ZFP179, 182, 245, 252* and *36* improved drought tolerance in transgenic rice through oxidative and antioxidant defence in an ABA-dependant manner (Sun *et al.,* 2010b; Zhang *et al.,* 2012; Zhang *et al.,* 2014). Two *BBX* genes from *Arabidopsis*, *BBX5* and *BBX24* (also called *AtCOL4* and *STO*), have been linked to osmotic stress tolerance (Min *et al.,* 2015; Nagaoka and Takano, 2003) and *MdBBX10* from apple enhanced drought stress tolerance in transgenic *Arabidopsis* (Liu *et al*., 2019). Similar, in the promoter regions of the tomato genes *SlBBX7* and *SlBBX12,* ABA responsive elements were identified, which most likely also indicate in link to drought responses (Chu *et al.,* 2016a).

Available knowledge suggest that BBX proteins can control stress responses in plants through stress-induced signalling. For example, some BBX genes are associated with modulating plant tolerance responses by means of hormone signalling and repression of brassinosteroids (BR) signals (Fan *et al.,* 2012; Luo *et al*., 2010; Sun *et al*., 2010a), targetting genes involved in the biosynthesis of osmotic adjustment substances and antioxidant genes associated with reactive oxygen species (ROS) scavenging (Han *et al.,* 2020). BBX proteins can manipulate these processes through DNA binding in mediating transcriptional regulation, RNA binding or protein-protein interactions (Gangappa *et al*., 2014).

In this study, the complete coding sequence of the *BBX29* gene from *Arabidopsis* was obtained from a pJET1.2 vector construct, which was provided by Prof SK Panda, India and subsequently cloned into a linearized pUBI510 plant expression vector (Figures 2.1 and 2.2). This recombinant vector was used to transform sugarcane calli and a number of putative genetically transformed plantlets were regenerated *in vitro* through particle bombardment and somatic embryogenesis. Three transgenic clones were confirmed to contain and express the *BBX29* transgene (Figures 2.3 and 2.5).

Under optimal conditions, overexpression of *AtBBX29* in sugarcane did not change the phenotype of transgenic plants with all genotypes displaying similar growth patterns to WT plants (Figure 2.6). Growth was assessed towards the end of the grand growth phase, after 8 months of growth, when cane formation and elongation responsible for yield has been accomplished (Ramesh, 2000). Carbohydrate levels in transgenic plants also remained largely unchanged with only some of the transgenic lines displaying lower glucose and fructose levels in mature cane tissue (Figure 2.7). Sucrose, the main product of sugarcane cultivation, serve as energy source for plant growth and can become a protective molecule when sugarcane encounter osmotic stress (de Maria Felix *et al.,* 2009). Studies have shown that continous growth and development of sugarcane internodes results in a gradient of maturation and sucrose accumulation. As the internode develops, the concentration of sucrose accumulation in the basal internode is higher than developing internodes above (Carson and Botha, 2002), which was also seen in this study where the mature internode tissue of all transgenic and WT plants had around 10x higher sucrose concentrations than those in the young immature stalk tissue (Figure 2.8). Depending on the ability of a

sugarcane cultivar to produce sucrose, the matured stalks of commercial sugarcane varieties accumulates between 600 to 700 mM of sucrose after a 12 month growth period, which is up to 60% of culms dry weight (Casu *et al*., 2004; Moore, 1995; Pereira *et al*., 2017; Verheye, 2010; Zhu *et al*., 1996). The transgenic plants overexpressing *AtBBX29* as well as the WT plants accumulated around 100 µmol sucrose per gram fresh weight after eight months of growth in internode nine tissue.

AtBBX29 overexpression enhanced tolerance to drought with delayed morphological damage and increased survival observed in the transgenic sugarcane plants exposed to water-deficit stress (Figure 2.9). Transgenic plants also recovered better from the stress compared to WT plants. This was regardless of the confirmation that plants were under severe water-deficit stress as indicated by the decrease in the RWC. Relative water content was measured to indicate the relationship between the water in the soil and water that was lost by transpiration (Anjum *et al.,* 2011). During the 21 days ww, the soil moisture content declined to almost completely dry soil in all pots included in the trial (Figure 2.10). During the same period, transgenic plants overexpressing *AtBBX29* and WT plants lost between 35-40% of their RWC. According to Hsiao, (1973) a 40% loss in water content indicate that plants are under severe osmotic stress.

Sugarcane is a crop with C4 photosynthetic metabolism, and under water-defict stress, photosynthetic performance are known to be negatively effected (Ferreira *et al*., 2017). Photosynthetic performance is directly linked to shoot biomass and finally crop yield. In this study, the photosynthetic performance of the transgenic sugarcane was maintained at higher levels than that in the WT throughout the drought period. Photosynthesis is mainly influenced through adjustments in stomatal conductance and chlorophyll mantainance.

Under non-stressed conditions all plants types showed Fv/Fm values of 0.75, which then rise slightly to 0.78 in some genotypes during the early days of water-deficit stress (~day 5 ww)(Figure 2.15 a). Fv/Fm is used as a sensitive indictor of plant photosynthetic performance and reflects maximum quantum yield of PSII whereby a change in the efficiency of non-photochemical quenching result in changes in Fv/Fm values (Bjorkman and Demmig, 1987). Optimal Fv/Fm values of around 0.83 are measured for most plant species and lower values indicate that a plant has been exposed to stress (Maxwell and Johnson,

2000). In sugarcane, Fv/Fm values are known to fluctuate around 0.78 under well-watered non-stressed conditions but can drop to 0.74 and lower under waterlogged or dry conditions (Soleh *et al*., 2018). In this study, slightly lower chlorophyll fluorescent values (0.75), measurements on day 0 of the trial, might indicate that the soil was waterlogged and needed to drain from the pots before plants were stress free and could display optimal Fv/Fm values (0.77 to 0.81 by ~ day 5 ww). Over time the fluorescence rate decreased in all plant types which is a photo protective mechanism resulting in lowered energy pressure in the PSI and PSII systems under conditions of excess energy (Zheng *et al.,* 2009), as also seen in sugarcane genotypes exposed to water-deficit stress (Gonçalves *et al*., 2010; Rodrigues *et al.,* 2009). By the end of the drought trial all transgenic lines maintained higher chlorophyll fluorescence rates, with lines, T1.8 and T1.1 obtaining significantly higher Fv/Fm values than WT plants.

A decrease in the PSII reaction can also be caused by stomatal closure as it limits leaf internal CO_2 concentrations (Lawlor and Terza, 2009). At the start of the drought trial, stomatal conductance was similar in all plants (Figure 2.15 b) but after 10 days ww the stomatal conductance of WT plants decreased while all transgenic lines overexpressing *AtBBX29* remained significantly higher throughout the drought stress period. In sugarcane, stomatal closure is a common tolerance mechanism that prevents transpiration (Inman-Bamber and Smith, 2005). It is known that stomatal closure is induced by an early ABA response, which is one of the important signalling hormones involved in water stress where it conserves the plants water status through restricting water loss during periods of low water availability in the soil (Basnayake *et al.,* 2015; Nayyra and Gupta, 2006; Ouyang *et al*., 2017; Yang *et al.,* 2006). Abscisic acid is a plant hormone produced in the roots of plants in reponse to decreasing soil moisture and then translocated to the plant leaves where it adjust the osmotic potential of stomatal guard cells, causing the stomata to close which in turns reduce water loss due to transpiration. Evidence of the role of BBX proteins in hormonal signalling pathways has been shown but is still poorly understood (Gangappa and Botto, 2014; Chu *et al*., 2016b). In this study, the level of ABA varied substantially between individual plants and genotypes across the dry period. No trend regarding the ABA levels at the different measure points in the different genotypes could be concluded, most likely due to technical experimental errors (Figure 2.16). A study by Li *et al*. (2016) reported a rise in

ABA content while stomatal conductance and transpiration rate declined gradually when sugarcane was exposed to increasing water-deficit.

Furthermore, dry conditions are known to accelerate chlorophyll degradation accompanied by an inactivation of photochemical reactions mediated by photosystem II (PSII). Plants use chlorophyll as a pigment for photosynthesis and it is known to decrease when water-deficit becomes severe in plants (Kuroda *et al.,* 1990; Muller *et al.,* 2011). In this study the decrease in the chlorophyll content of WT plants did not differ significantly from that of the transgenic plants, only slightly higher levels of chlorophyll was seen in the transgenic plants under mild (days 7 and 14 ww) water-deficit conditions. However, the chlorophyll content was measured in the TVD leaf of the plants, this is one of the younger leaves on the plant, while leaf chlorosis, indicative of chlorophyll degradation and senescence, was clearly enhanced in the majority of mature leaves in the WT plants compared to the transgenic plants, as indicated by the number of damage plants in Table 2.3. As reviewed by Liu *et al.* (2008), sugarcane leaf senescence is a natural process that shows maturation and aging of the leaves, chlorophyll degradation and recycling of nutrients to stalk tissue (Franco *et al.,* 2013; Martins *et al.*, 2016). Transgenic plants able to delay this process of senescence can avoid limitations in crop yield and biomass losses and would be a great benefit to the sugarcane industry (Guo *et al.,* 2004; Liu *et al*., 2008). Transcriptome analysis revealed that zinc finger types C2H2, C2C2 and C3H are among genes associated with leaf senescences in *Arabidopsis* (Guo *et al*., 2004).

Additionally, during water-deficit stress conditions, a wide range of free amino acids acummulates in high concentrations to provide protection to plants (Yadav *et al*., 2019). Reports refer to some of these amino acids, such as proline, as compatible solutes or osmoprotectants, which contribute to cellular osmotic adjustment (Ashraf and Harris, 2004; Serraj and Sinclair, 2002). Transgenic crops with increased proline levels and high osmotic potential displayed reduced water-loss, stable subcellular structure and tolerance to osmotic stress (Efeoğlu *et al.,* 2009; Hong *et al.,* 2000; Reddy *et al*., 2015; Saxena *et al.,* 2013). A study by Hayat *et al*. (2012) and Molinary *et al.* (2007) reported that in transgenic sugarcane instead of osmotic adjustment, increased proline accumulation enhanced biomass yield and photoquemical efficiency of photosystem II. In this study, a reflection of osmotic adjustment as a response to water-deficit stress was seen with significantly

increased proline levels in transgenic plants overexpressing *AtBBX29* when water-deficit was mild (day 14 ww) and severe (day 21 ww). Similar outcomes were reported as improved drought tolerance when transgenic wheat was exposed to 14 days of water stress (Sawahel and Hassan, 2002). Other studies, suggested increased accumulation of proline led to improved tolerance to drought based on parameters such as total water potential, osmotic and recovering abilities (Chen *et al*., 2016; Hayat *et al*., 2012; Molinari *et al.*, 2007; Reddy *et al.,* 2015; Serraj and Sinclair, 2002). Similarly, overexpression of the zinc finger *ZFP252* gene in rice enhanced salt and drought tolerance in transgenic plants with higher proline content, with plants displaying enhanced transcript levels of *OsP5CS* and *OsProT* (encode proline transporter), and other stress-related genes such as *Oslea3* (late embryogeneis abundant) which is associated with the accumulations of osmoprotectants when plants undergo dessication (Xu *et al.,* 2008). *AtBBX29* may be one of the upstream regulators of these genes mediating expression for accumulation of campatible osmolytes in sugarcane under drought stress.

In addition to osmotic adjustment, proline also acts as an osmolyte to stabilize enzymes/proteins, protect membrane integrity and detoxifing reactive oxygen species (Ashraf and Foolad, 2007; Bohnert and Jenhsen, 1996; Yancey *et al.,* 1982). Hemaprabha *et al.* (2013) also suggested that plants use proline as nitrogen and carbon to recover their physiological activities after stressful conditions. Previous studies in sugarcane suggested that accumulation of high proline content provided both actions as an osmoregulator and an antioxidant when water was limited (Cia *et al.,* 2012; Hemaprabha *et al.,* 2013; Molinary *et al*., 2007).

Plants naturally produce reactive oxygen species (ROS) during normal metabolic processes such as photosynthesis (Hossain *et al.,* 2015). While hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^{1}O_2$) are kept at minimum concentrations, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced at higher rates and plays crucial roles as signalling molecules (Gill and Tuteja, 2010; Jakob and Heber, 1996). However, under water-deficit conditions a considerable increase of these molecules becomes a toxic by-product of oxidative stress where it destroy cellular components such as DNA, membrane lipids and proteins (Anjum *et al.,* 2011; Ashraf, 2009; Gill and Tuteja, 2010; Xie *et al.,* 2019). In this study the transgenic

plants showed significantly lower levels of ROS accumulation than WT plants during the 21 days of water-deficit (Figure 2.12 a).

In a recent review conducted by Han *et al*. (2020), C2H2 zinc finger proteins may be involved in maintaning stable ROS levels through directly targeting expression of antioxidase genes linked with removal of ROS (Gadjev *et al.,* 2006). This was confirmed in a study by Rizhsky *et al.* (2004) in which they showed that upon oxidative stress, *ZAT12* overexpression in *Arabidospsis* elevated the expression of an *ascorbate peroxidase 1* (*Apx1)* gene known to be an H_2O_2 scavenger (Davletova *et al.,* 2005; Rizhsky *et al.*, 2004). *ZFP245* overexpression in rice enhanced drought resistance by increasing ROS scavenging, where transgenics displayed enhanced activity of ROS scavenging enzymes (Xu *et al.,* 2008). Additionally, a previously unknown zinc finger TF named *DST* (*drought and salt tolerance*), was seen to control levels of H_2O_2 via ABA-independent pathways in *Arabidopsis* where the expression of *DST* regulated expression of a *peroxidase 24 precursor* gene, which is one of the associated ROS homeostasis-related genes, which when repressed give rise to H_2O_2 accumulation and stomatal closure (Huang *et al.,* 2009). Thus, *AtBBX29* overexpression in sugarcane may have contributed to regulation of downstream responsive genes which function in maintaining ROS homeostasis.

In this study, antioxidant activity of superoxide dismutase (SOD) and catalase (CAT) were measured in response to water-deficit stress. Studies have reported increased levels of these enzymes influence stress tolerance in plants where SOD constitutes the first enzyme responding against oxidative stress in most cellular compartments. SOD catalyzes the dismutation of O_2^- and generate oxygen and H_2O_2, which is further decomposed by CAT into water and oxygen (Mittler, 2002 Mhamdi *et al*., 2010). Here we report significantly higher SOD and CAT levels in the *AtBBX29* overexpressing lines than what was seen in the WT plants at the end of the 21 day water-deficit stress period (Figure 2.14). Studies showed that the activity of CAT and SOD in sugarcane is genotype dependent, where increased activity are mostly displayed in sugarcane cultivars tolerant to water-deficit (dos Santos *et al.,* 2015; Hemaprabha *et al*., 2012; Janpromma *et al*., 2012; Sales *et al.,* 2013). In sugarcane, these enzymes regulating redox levels in cells increases the capacity of sugarcane cultivars to eliminate the toxicity of H_2O_2 under limited water conditions (dos Santos and de Almeida

Silva, 2015). Another H_2O_2 scavenging molecule, reduced glutathione (GSH) activity was not significantly different between transgenics and WT plants (Figure 2.14 d).

From these results we can deduce that overexpression of the *AtBBX29* gene may play a role in increased antioxidant enzyme activities under drought stress. Other studies have showed that under abiotic stress, antioxidase genes known to be involved in ROS scavenge are directly regulated by expression of C2H2-type zinc finger proteins resulting in enhanced drought resistance (Liu *et al*., 2017). Overexpression of *ZFP245* and *ZFP179* in rice increased activities of SOD and peroxidase in response to a multiple of stress conditions and resulted in increased stress tolerance (Huang *et al*., 2009; Sun *et al.,* 2010b). Furthermore, the *Zat12* gene in *Arabidopsis* upregulated the expression of the *APX1* antioxidant defence systems under light stress (Davletova *et al.,* 2005). Ascorbate peroxidase (APX) is a H_2O_2 decomposing enzyme. Expression of the *ZFP36* gene resulted in higher SOD activity. These findings also suggest that overexpression of *AtBBX29* gene may have played a similar role in targeting the expression of antioxidant genes for removal of ROS and enhanced protection of transgenic sugarcane from oxidative membrane damage. However, this will need to be confirmed in the future by analysing transcript levels of a number of relevant ROS and antioxidant scavenging genes.

The increased activity of SOD and CAT in transgenic lines during the dry period can be linked to the decrease in H_2O_2 and MDA concentrations compared to the WT plants. Malondialdehyde (MDA) is a marker of oxidative stress and results from lipid peroxidation, often used as an indicator of damage of membrane lipids (Xie *et al.,* 2019). In the current study, the MDA levels observed in transgenic plants was significantly lower in most of the transgenic lines compared to the levels observed in WT plants after 21 days ww (Figure 2.11). Low levels of lipid peroxidation have been associated with cell survival through continous activation of signalling pathways, which results in adaptation and higher antioxidant ability, reflecting increased resistance to water-deficit stress. The maintenance of a healthy phenotype for extended periods within the water-deficit period in the transgenic plants might therefore be ascribed to less oxidative damage, lower accumulation of ROS due to an efficient anti-oxidant scavenging system. Similar results were reported on overexpression of *PtrZPT1-2* where transgenics displayed increased survival rates and

osmolytes but decreased levels of MDA and reduced H_2O_2 accumulation in transgenic tobacco under drought stress (Liu *et al.,* 2017).

To conclude, the increased activity of SOD, CAT and proline in all transgenic lines during the dry period can be linked to the decrease in H_2O_2 and MDA concentrations compared to the WT plants. The maintenance of a healty phenotype and continues photosynthesis for extended periods within the water-deficit preriod in the transgenic plants might therefore be ascribed to less oxidative damage, lower accumulation of ROS due to an efficient antioxidant scavenging system.

Cellular localization studies conducted by Chu *et al*. (2016a) reported that the majority of tomato B-box zinc finger (BBX) proteins had a high probability to be localized to the nucleus. However, some, such as *SlBBX5, 6, 23* and *28* and *SlBBX16*, 18 and 19*,* could be located in different subcellular compartments (cytosol, chloroplast etc.) based on software predictions. Information extracted from the Arabidopsis information (TAIR) database (https://www.arabidopsis.org) predicted that *BBX29* is most likely localized to the nucleus (SUBA: Subcellular localization database for Arabidopsis proteins; http://suba.live/factsheet.html?id= AT5G54470.1). However, locations such as the cytosol and mitochondria for *BBX29* localization was also predicted by the software. The *AtBBX29* subcellular localization experiment was initiated by fusing the *AtBBX29* protein upstream of the green fluorescent protein (*GFP*) reporter gene in the pCambia1302 expression vector. However, fluorescence signals in the transformed onion epidermal cells were weak and could correspond to auto fluorescence and poor *GFP* expression (Figure 2.19). This experiment was unsuccessful and will have to be repeated since no fluorescence was obtained that could to be associated with *GFP* and localization of the *AtBBX29* gene.

CHAPTER 3: OVEREXPRESSION OF A TOMATO *NAC2* GENE IN SUGARCANE TO FUNCTIONALLY ANALYSE ITS ROLE IN DROUGHT TOLERANCE

3.1 INTRODUCTION

Drought is a universal environmental constraint which negatively influence the sustainable production of many crop species, including sugarcane (Fahad *et al.*, 2017; Vadez *et al.*, 2008; Silva *et al.,* 2007). Drought occurrence is often unpredictable and seasonal drought periods cannot be avoided in sugarcane cultivation since its growth period span across many months (Edossa *et al.,* 2014). Over the past few years, sensitivity of this crop to severe drought has resulted in harvest and plantation losses in a number of cultivation areas worldwide (Inman-Bamber *et al.,* 2012). Therefore, it is important to understand the mechanisms involved in plant tolerance to abiotic stress which can lead to the production of water-deficit stress tolerant sugarcane varieties (Hussain *et al.*, 2008).

Genetic engineering targeting multigene regulatory pathways might result in the expression of proteins enhancing stress tolerance in plants through changes in the plant's physiology and biochemical processes (Huang *et al*, 2012; Hussain *et al.,* 2012; Li *et al,* 2019). Transcription factors (TFs) are proteins that regulate the transcription of multiple genes by binding to *cis*-elements in their promoter regions, thereby activating potentially several downstream genes responsive to abiotic stress (Century *et al.*, 2008; Wang *et al*, 2016c).

No apical meristem; ATAF1/2 and CUC2, cup-shaped cotyledon (NAC) TFs are part of a large plant specific family of TFs, which regulate a great number of genes annotated to contribute in the regulation of drought resistance (Naruzzaman *et al.*, 2010). No apical meristem; ATAF1/2 and CUC2, cup-shaped cotyledon regulated genes are structurally recognized by a NAC domain which are highly conserved at the N-terminal for DNA binding (~150 amino acids) and a diversified C-terminal for activation of transcription (Christianson *et al.,* 2010; Ooka *et al.,* 2003; Redillas *et al.,* 2012; Shao *et al.,* 2015). Genome wide transcriptome analysis predicts that up to 25% of NAC genes function in plant stress responses (Fang *et al.,* 2008; Naruzzaman *et al.,* 2010).

In tomato (*Solanum lycopersicum L)*, genome wide association studies identified 104 NAC TFs and analyzed their expression patterns under various stress conditions (Su *et al.,* 2015). To date some of these tomato NACs have been functionally annotated. *SlNAC4 (*Zhu *et al.,* 2014), *SlNAC19* and *SlNAC48* (Kou *et al*., 2016), *SlNAC3* (Gao *et al*., 2008), *SlNAP2* (NAC-like activated by Apetala3/Pistillata; Ma *et al.,* 2019) and *NOR* (NON-RIPENING; Ma *et al.,* 2019) are all implicated in the control of leaf senescence and fruit ripening, by affecting ethylene biosynthesis and signalling, and carotenoid accumulation. *SlNAM2* NAC TF was shown to be involved in flower whorl and sepal boundaries (Hendelman *et al.,* 2013). Previous studies also linked a number of tomato NAC TFs in biotic and abiotic stress regulation. When *SlNAC35* was overexpressed in tobacco plants, transgenic plants showed improved root growth by enhancing expression of *AUXIN RESPONSE FACTOR* (*ARF*) genes and improved defence mechanisms against bacterial pathogens through a salicylic acid (SA) signalling pathway involved in reactive oxygen species (ROS) mediated hypersensitivity responses (Wang *et al.,* 2016b). The *SlSRNI* tomato NAC TF conveyed resistance against biotic stresses but decreased tolerance towards drought stress (Liu *et al.,* 2014a), while the expression of *SlNAC3* was also inhibited by salt, drought and ABA treatment (Han *et al.,* 2012).

Recently, *SlNAC2* was proven to be effective towards improving drought stress tolerance in transgenic tobacco plants. Overexpression of this gene accelerated flowering and caused a delay in leaf senescence under severe drought due to the expression of stress responsive genes, among which *delta 1-pyrroline-5-carboxylate synthase* (*P5CS),* associated with regulating osmo-protectant accumulation, anti-oxidative defence and water retention in plants, resulted in improved drought tolerance (van Beek, 2018). The same NAC gene in transgenic *Arabidopsis thaliana,* exposed to osmotic stress, resulted in the regulation of antioxidant defence mechanisms by upregulating *glutamyl cysteine* (-EC) and *EC synthase* (ECS) in the glutathione (GSH) pathway, which takes part in ROS detoxification (Borgohain *et al.,* 2019). It is clear from these reports that the *SlNAC2* gene plays an important role in tolerating water-deficit stress in model plant species. However, the question remains whether this TF will also contribute towards drought tolerance in a crop species such as sugarcane. Therefore, in this study, the *SlNAC2* gene from tomato was overexpressed in sugarcane to determine its functional involvement in enhancing drought tolerance in this crop species.

3.2 MATERIALS AND METHODS

3.2.1 Transgene

The full length *SINAC2* TF gene (960 bp), isolated from tomato (*Solanum lycopersicum* L; GenBank: KT740994.1; Borgohain *et al*., 2019), was provided by Prof SK. Panda, Assam University, India. The gene was isolated from tomato roots, cloned into the pJET1.2 vector, transformed into *E.coli* and send to us as a culture stock.

3.2.2 Construction of a plant expression vector

The full length *SINAC2* gene was amplified in a standard PCR reaction using GoTaq polymerase (Promega), from pJET1.2:*SINAC2* vector template, and a gene specific primer pair (Table 3.1) containing *Bam*HI restriction sites. The thermal cycler conditions was set at an initial denaturation of 95^{o}C for 3 min followed by 35 cycles at 95^{o}C for 30 s, 60^{o}C for 30 s and 72^{o}C for 40 s, and a final extension at 72^{o}C for 5 min followed by a decrease in temperature to 4^{o}C. After digestion with *BamHI* (New England Biotechnologies (NEB); CutSmart buffer at 37°C), the 964 bp amplicon was separated through electrophoresis on a 1% (w/v) agarose gel prepared in TBE buffer (5.4 g/L Tris-base, 2.75 g/L boric acid and 0.465 g/L ethylenediaminetetraacetic acid (EDTA); pH 8). The amplicon was visualized under UV light using a G:BOX gel documentation system (Syngene, SDI Group, Cambridge, UK) and then gel purified using the Wizard® SV Gel and PCR Clean-Up Kit (Promega). In addition, the plant expression vector pUBi510 was linearized with BamHI and the *SINAC2* gene amplicon ligated into this vector and transformed using the standard heat shock protocol into *E. coli* cells (described in section 2.2.3.2) and selected on LB medium containing 50 mg/L ampicillin. The pUBi510 vectors contain an ubiquitin promoter for constitutive gene expression and a cauliflower mosaic virus (CaMV) terminator. PCR was used to screen putative transformed bacterial colonies for transgene integration and orientation using GoTaq and a combination of transgene specific and vector primers (Table 3.1). Transformed recombinant DNA clones were sequenced at the Central Analytic Facility (CAF) at Stellenbosch University, and

analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI), to confirm transgene integrity.

3.2.3 Plant transformation and tissue culture

Transgenic plant lines from *Saccharum* species hybrid cv. NCo310 were created by particle bombardment of embryogenic callus. Callus was initiated from immature apical inner leave roll segments of sugarcane stalks material and bombardment procedures carried out according to the method described by Birch and Bower (1994) with modifications as described in section 2.2.4.

The DNA precipitation mix was prepared containing 5 mg tungsten (Grade M10: Biorad, #165-2266) sterilized in 100% ethanol and rinsed 3 times with sterile water. The tungsten was mixed with 5 µl of the pUBi510:*SINAC2* (1 µg/µl) and pEmuKN (1 µg/µl) vectors carrying the transgene and *neomycine phosphotransferase II (nptII)* selection marker gene, respectively. Precipitation of the DNA mix were completed with the addition of 50 µl 2.5 M $CaCl_2$ and 20 µl 100 mM spermidine, while keeping the solution on ice. Prior to bombardment, tissue preparation and bombardment conditions were set up as described in section 2.2.4. Two days after bombardment, tissue was transferred to selection media (MSC3 containing 45 mg/L geneticin), described in section 2.2.5.4, for 8 weeks in the dark at 26^{o}C with sub-culturing onto fresh MSC3-G45 medium every second weeks. Putative transgenic callus clones, surviving the selection phase, were transferred to semi-solid MSC growth media, lacking 2,4-D and incubated at 26^{o}C at a 16/8-hour light/dark photoperiod under cool white fluorescent lights to allow somatic embryo formation. Cultures were sub-cultured every other week onto fresh culture media. Generated plantlets were allowed to form roots and grow to a height of ~ 5 cm.

Plants with roots were hardened off in 20 cm pots containing a soil mixture consisting of 2-parts palm peat; 1-part sand; and 1-part vermiculite. Plantlets were covered with a plastic bag to prevent the plants from drying out during the hardening off phase. Pots were placed in the greenhouse under natural light conditions at temperatures around 26 ± 2^{o}C.

3.2.4 Confirmation of transgene integration and expression

Genomic DNA from each putative transformation clone and wild-type control sugarcane was extracted from 150 mg leaf tissue using the ZR Plant/Seed DNA MiniPrep Kit (Zymo Research). Transgene integration was confirmed through standard PCR using GoTaq (Promega) and 200 ng of gDNA as template in combination with gene specific and ubiquitin promoter primer pairs (Table 3.1). PCR reactions were supplemented with 1.5 mM $MgCl_2$ and conditions were set as follows: initial denaturation 95°C for 3 min followed by 35 cycles of denaturing at 95°C for 30 s, primer annealing temperature of 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min followed by a decrease in temperature to 4°C. Amplicons were separated and visualized through gel electrophoresis as described previously.

Total RNA was extracted using the Maxwell® 16 LEV Plant RNA Kit (Promega) from 80 mg leaf tissue harvested from transgenic and wild-type control plant lines. cDNA was then synthesized from 1 µg RNA using the Reverse Aid H minus First strand cDNA synthesis Kit (ThermoFisher Scientific). Semi-quantitative reverse transcriptase PCR (sq-RTPCR) was performed using cDNA (1 µL) template, GoTaq DNA polymerase and transgene specific primers (Table 3.1). PCR was performed using standard settings with a primer annealing temperature of 60°C. *Actin* served as internal reference gene. Actin specific primers are listed in Table 3.1. The 600 bp amplicons were separated and analyzed on a 1% (w/v) agarose gel under UV light.

Table 3.1 PCR primers used for *SlNAC2* and *Actin* gene isolation and detection.

Primer name	Primer sequence (5'-3')	Function	Tm	Amplicon size
UbiNACBamHI Forw	GCAGGTCGACGGATCGTAATGGAAA TTGTGTGTGG	Gene amplification	60°C	600 bp
UbiNACBamHI Rev	GAATTCCCGGGGATCATATCAATAAT TCCATAGACAATCG			
Ubi Prom Forw	ATACGCTATTTATTTGCTTGG	Gene orientation; vector confirmation	54°C	
NAC Forw	GCTTATAACACACTATTTAGCC	Gene amplification	55°C	500 bp
NAC Rev	TGGTGGCAAATTTGAAGACCT			
NAC RT Forw	GCAGGGTATTGGAAGGCAAC	Gene integration; expression	60°C	600 bp
NAC RT Rev	GTCATGATCTTCGTAACCTCTCA			
Actin Forw	TCACACTTTCTACAATGAGCT	Internal gene expression; cDNA quality	53°C	600 bp
Actin Rev	GATATCCACATC ACACTTCAT			

3.2.5 Drought pot trials

Independent transgenic and wild-type control sugarcane plantlets were multiplied *in vitro* and transferred to 20 cm pots containing a 2 kg mixture of soil (2:1:1 peat:sand:vermiculite). Sugarcane plants were allowed to grow for 4 months in the glasshouse at around 26 ± 2°C under natural light. Plants received water regularly and 3 g/L Hydrotech Generic Fertilizer (Hydrotech, Stellenbosch, SA) and 2.5 g/L calcium nitrate [$(Ca_2(NO_3)^2$] every 2 weeks.

After 4 months of growth, healthy plants in the tillering stage of development were deprived of water for 31 days followed by re-watering. For each genotype, 25 transgenic sugarcane and wild-type plants were assayed and readings taken at each time point from three biological replicates. Survival rates were determined after the stress period and 14 days after re-watering. Plants were photographed at days 0, 14, 21, 28 and 31 after withholding water (ww) and after recovery.

Physiological measurements were taken prior to and every second day after ww using the top visible dew lap (TVD) leaf as measuring point. For this, stomatal conductance was measured at three position on the TVD leaf according to the method described by Pask *et al*. (2012) using the Decan leaf Porometer SC-1 (Decagon Devices Inc. Pullman, USA). Similarly, chlorophyll fluorescence was measured according to the method described by Maxwell and

Johnson (2000) using the OS-30p+ (OPTI-SCIENCES, Hudson, USA) chlorophyll fluorometer. Stomatal conductance and chlorophyll fluorescence were expressed as mmol.m^2s^1 and Fv/Fm, respectively.

During the water stress period the soil moisture content of all pots included in the trial was measured every second day and three readings were taken per pot at a depth of 10 cm using the ProCheck soil moisture sensor probe (Decan Devices; Washington, USA). Three biological replicates were used for relative water content (RWC) measurements and root biomass on days 0, 14, 28 and 31 ww, where 1 cm length leaf discs, three disks per plant, were excised from the TVD leaf and the fresh weight (FW) was recorded. The turgid weight (TW) and dry weight (DW) were obtained after floating the leaves in deionized water for 2 days and drying the disks at 60^{o}C for 3 days. The roots were harvested and fresh weight was recorded. Dry weight was recorded after drying the roots at 80^{o}C for 2 days. Relative water content was calculated as a percentage using the formula:

RWC = [(FW – DW)/ (TW – DW)] x 100%

3.2.6 Protein subcellular localization

A full length *SINAC2* amplicon was generated through PCR containing the CACC recognition sequence using the Q5 High Fidelity DNA polymerase (NEB, Ipswich, MA, USA) and the pENTRY forward and reverse primer pair (Table 3.2). PCR conditions consisted of initial denaturation 95^{o}C for 3 min followed by 35 cycles of denaturing at 95^{o}C for 30 s, primer annealing temperature of 54^{o}C for 30 sec and 72^{o}C for 1 min with a final extension at 72^{o}C for 5 min. The 964 bp amplicon was recombined into the *attL* sites of an entry vector system provided by the pENTR™ Directional TOPO Kit (Invitrogen, Carlsbad, CA), following the manufacturer's instruction. The recombinant vector was transformed into OneShot®Top10 chemically competent *E. coli* cells, using a standard heat shock protocol and transformed cells were selected on LB (Lysogeny Broth) medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar; pH 7) containing 50 mg/ml kanamycin. Colonies were screened by PCR using GoTaq and a combination of gene specific and M13 forward and reverse vector primers to confirm gene insertion (Table 3.2), followed by sequencing to confirm transgene

integrity. Sequencing was done at the Central Analytic Facility (CAF) at Stellenbosch University.

Purified *SINAC2* was sub-cloned into the pGWB506 plasmid (Addgene vector #74848; Massachusetts, USA) Gateway® compatible destination using the LR recombination reaction with Clonase® as described in the Gateway ® Technology manual (Invitrogen, Carlsbad, CA). The recombinant vector was transformed into chemically competent *E. coli* OmniMAX cells prepared as previously described in section 2.2.3.2. The pGWB506 is a binary vector containing a fluorescent reporter *EGFP* (enhanced *GFP*) gene, cauliflower mosaic virus (CaMV) 35S promoter and *nopaline synthase* (*nos)* terminator. Positive colonies were selected on LB medium containing 100 mg/L spectinomycin and screened using GoTaq and a combination of gene specific and vector primers to confirm insertion (Table 3.2). The recombined sequence was further analyzed to confirm that the ATG (start codon) of the *EGFP* gene was in frame with the ATG of the transgene and without a stop codon in between (Supplementary data, Figure S10).

Recombinant vector DNA, pGWB506:*SINAC2* and pGWB506 were isolated using the GeneElute Plasmid Mini-prep kit (Promega, Medison, USA). Electro-competent *Agrobacterium tumefaciens* cells, strain LBA4404 (100 µl) were separately transformed with 100 ng DNA of each vector using the Gene Pulser Xcell electroporation system (BioRad Laboratories, USA) at 2500 V, 25 µF and 200 Ω in 0.2 mm gap cuvettes. Electroporated *Agrobacterium* cells were grown and selected on YEP medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L sodium chloride) containing 50 mg/ml rifampicillin, and 100 mg/L spectinomycin. Putative transformed *Agrobacterium* cells were screened by PCR using GoTaq and a combination of gene specific and vector primers (Table 3.2). Transformed *Agrobacterium* cells containing the recombinant DNA were mix with 80% (v/v) glycerol and flash-frozen in liquid nitrogen and stored at 80^{o}C till used.

Onion epidermis cells were infiltrated with transformed *Agrobacterium* cells as described by Xu *et al*., (2014) and outlined in section 2.2.10.3. After the co-cultivation period the region around the inoculation site were peeled from the onion scales and placed onto a droplet of water on the surface of a standard microscope slide and covered with a coverslip. Onion cells were visualized for *GFP* fluorescence using an Axio Scope. A1 microscope connected to

ZEISS Axiocam 305 colour imaging software. The cells were subjected to laser excitation of 520-550 nm at 4x and 10x magnifications and bright green field images were captured.

Table 3.2 PCR primers used for *SlNAC2* cloning into the pGWB506 destination vector.

Primer name	Primer sequence (5'-3')	Function	Tm	Amplicon size
pENTRY Forw pENTRY Rev	CACCATGGAAATTGTGTGTGGA TCAATAATTCCATAGACAATCG	Gene amplification; confirmation	54°C	964 bp
M13 Forw M13 Rev	GTTTTCCCAGTCACGAC CAGGAAACAGCTATGAC	Confirm gene insertion; orientation in D-TOPO entry vector	45°C	1302 bp
GFP Forw *GFP* Rev	CATGGTGAGCAAGGGCGA TGCTCAGGTAGTGGTTGCG	Confirm reporter gene	60°C	600 bp

3.3. RESULTS

3.3.1. Transgene cloning into the plant expression vector

Through PCR the full length coding sequence of the *SINAC2* gene, was amplified from the pJet1.2:*SINAC2* vector provided by Prof SK Panda, India. The amplicon was isolated and cloned into the pUbi510 plant expression vector. PCR analysis confirms that *E. coli* colonies (1-9) were successfully transformed with the pUbi510:*SINAC2* recombinant vector construct (Figure 3.1).

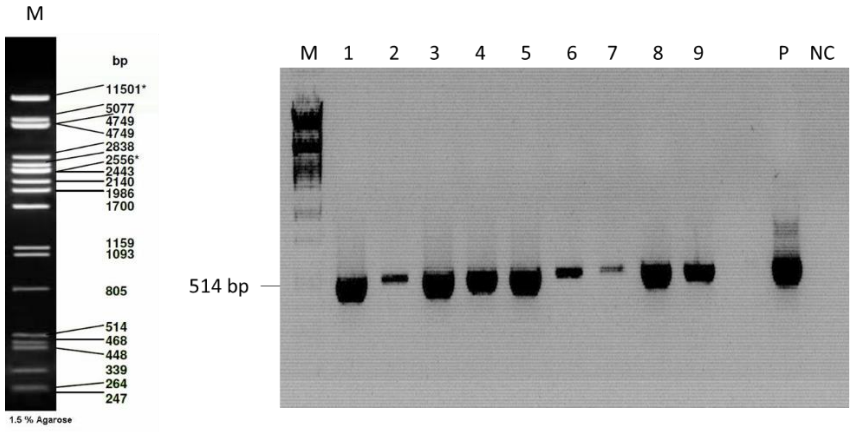

Figure 3.1. Colony PCR, using the Ubi Prom For and NAC Rev primers, confirming cloning of the *SINAC2* gene into the pUbi510 plant expression vector and transformation into the *E. coli* DH5α cells. Lanes M (*Pst*I λ marker); 1-9 (transformed *E. coli* colonies); P (positive plasmid control; pJet1.2:*SINAC2*); and NC (negative H_2O control).

3.3.2 Confirmation of sugarcane transformation with the *SINAC2* transgene

Sugarcane embryogenic calli were regenerated, bombarded with the *SINAC2* gene and five putative transformed clones regenerated via somatic embryogenesis (Figure 3.2).

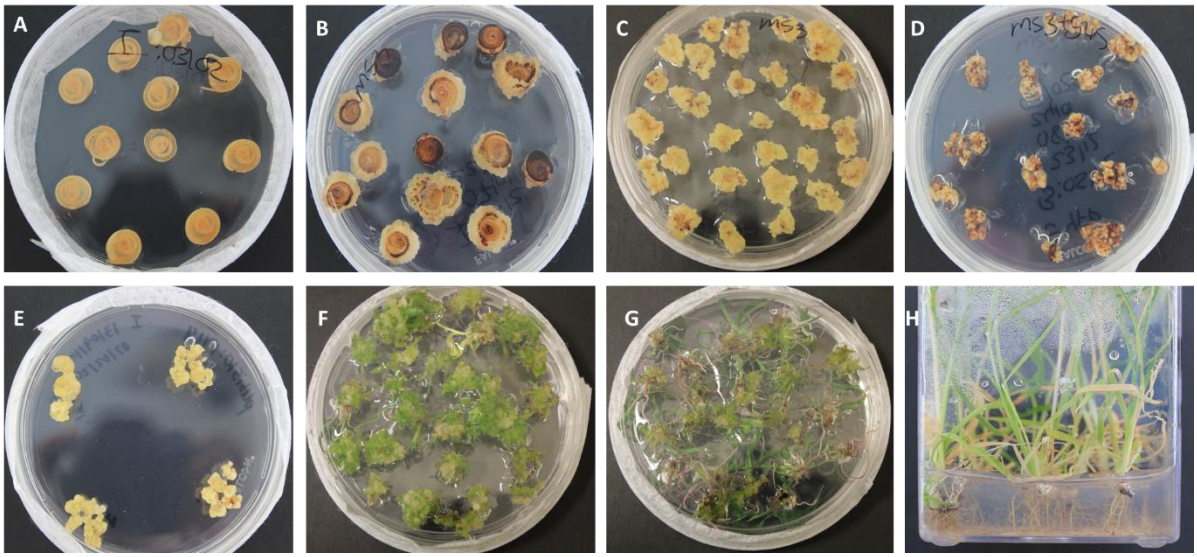

Figure 3.2. Generating *in vitro* putative transformed sugarcane (*Saccharum* spp hybrid cv. NCo310) plantlets from bombarded embryogenic callus. Callus was initiated from **(a)** immature inner leave roll explants isolated from stalk material; followed by the **(b)** development of callus and the harvesting of embryogenic callus **(c)** prior to transformation. **(d)** Bombarded callus was placed on selection media, and surviving calli **(e)** were allowed to develop somatic embryos **(f)**, which developed into sugarcane plantlets **(g)** that were allowed to elongate and form roots **(h)**.

Five putative transformed sugarcane events, lines T1.1, T1.2, T1.3, T1.4 and T1.5, were regenerated and analyzed to confirmed the presence of the *SINAC2* transgene using PCR and gene specific primers. For this, genomic DNA (gDNA) extracted from the leaves of putative transformed sugarcane and WT plants was used as template. An amplicon (600 bp) was successfully obtained from the genome of all 5 transgenic lines, while no amplification was detected in the WT plant (Figure 3.3).

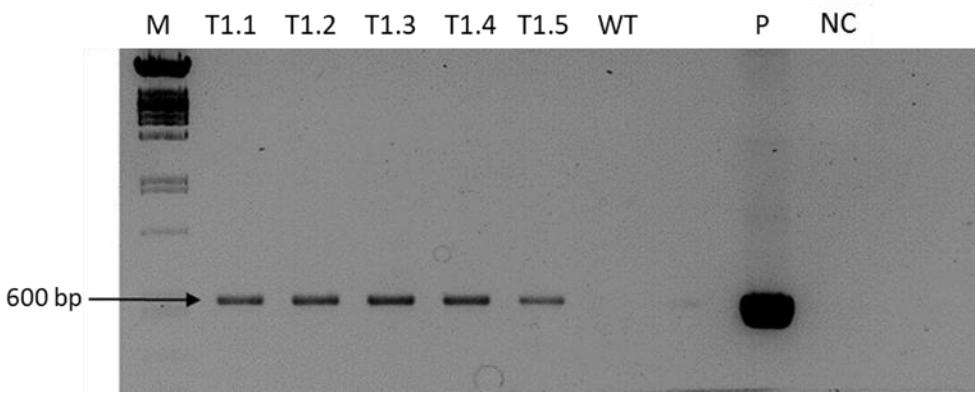

Figure 3.3 PCR analysis confirming the presence of the *SINAC2* transgene in five of the putative transformed sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5). M (*Pst*I λ marker); P (positive plasmid control; pJet1.2:*SINAC2*); and NC (negative H_2O control).

3.3.3 Analysis of transgene expression in transgenic sugarcane

The transformed sugarcane lines containing the *SINAC2* transgene were further analysed for the relative expression of the inserted gene through semi-quantitative reverse transcriptase-PCR (sqRTPCR). RNA was extracted from leaf material from the transgenic and WT plants (Figure 3.4) and cDNA synthesized. Gene expression levels were assessed by amplifying a 600 bp fragment using PCR primers specific to the *SINAC2* gene (Figure 3.5 b) and the *Actin* reference gene (Figure 3.5 a). Gene expression levels obtained from the analysis indicated that *SINAC2* was successfully expressed in all 5 transgenic lines. Three transgenic lines, T1.1, T1.2 and T1.4, showed relative high transgene expression levels, while relative low transcript levels of the *SINAC2* transgene were detected in T1.3. No expression was detected in the WT plants (Figure 3.5 a).

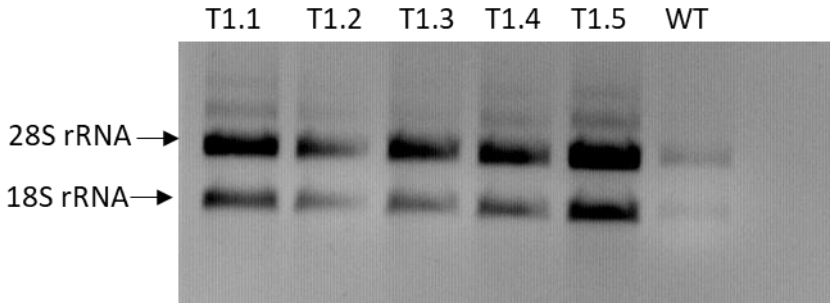

Figure 3.4. Gel electrophoresis of RNA extracted from the leaves of the *SINAC2* transformed sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5) and WT plant. RNA separation was conducted on a 1% (w/v) TBE agarose gel, run at 100 V.

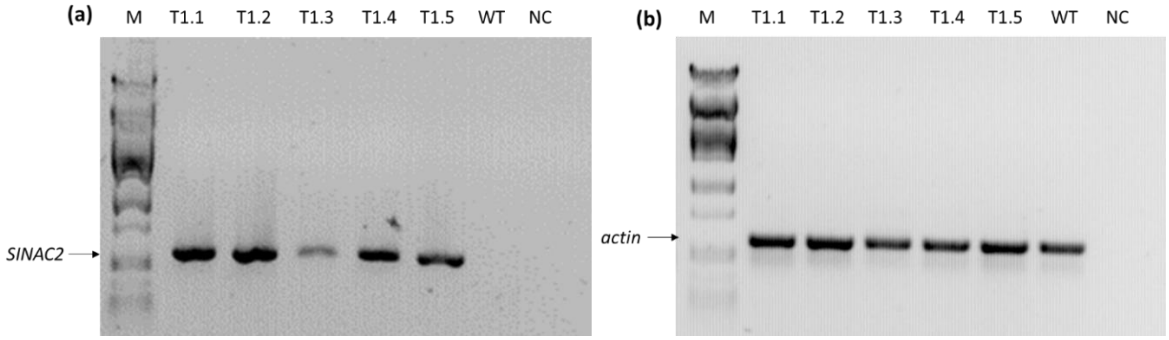

Figure 3.5. Semi-quantitative reverse transcriptase-PCR (sqRTPCR) showing expression levels of the *SINAC2* transgene in transgenic sugarcane lines (T1.1, T1.2, T1.3, T1.4 and T1.5) and WT plants using **(a)** gene specific primers in comparison to **(b)** *actin* as an internal control. M (*Pst*I λ marker); and NC (negative H_2O control).

3.3.4 Phenotypic response of WT and transgenic sugarcane exposed to drought

Four months old *SINAC2* transgenic sugarcane, lines T1.1, T1.2, T1.5, and WT plants were exposed to 31 days of water-deficit stress. The stress period were further extended to 35 days ww to increase the pressure on the plants. The plants were monitored for their phenotypic response and survival rates during and after the drought stress period. At the start of the water-deficit stress period (day 0 ww) and the following 14 days, the different sugarcane genotypes were morphologically all the same and showed no significant differences in growth, which included measurements of plant height, TVD length and width (Figure 3.6; Table 3.3). Even under severe extended water-deficit stress, no significant

differences were observed in the phenotype between the transgenic and WT plants. However, by day 21 ww most of the transgenic plants had TVD leaves that were significantly narrower that those formed on the WT plants (Table 3.3) and the WT plants started to display stress symptoms such as leaf wilting and chlorosis (Figure 3.6).

After 25 days ww, 17% of WT plants were dead with leaves and stalks being completely dry, brittle and brown. This is in contrast to all the *SINAC2* transgenic lines, of which 83 to 100% displayed no symptoms of water-stress at this time point (Table 3.4). Further extension of water-deficit to 31 days ww resulted in the death rate of the WT plants increasing to 43%, while only the T1.5 transgenic line also had plants dying (29%). A high percentage, between 67 and 78% of the plants of transgenic lines T1.1 and T1.2 were still healthy at this stage of the drought stress period. By day 35 ww, 75% of the wild-type plants were dead, in contrast only 25% of T1.1, 0% of T1.2 and 50% of T1.5 plants died, even though most of the transgenic plants started displaying symptoms of water-stress.

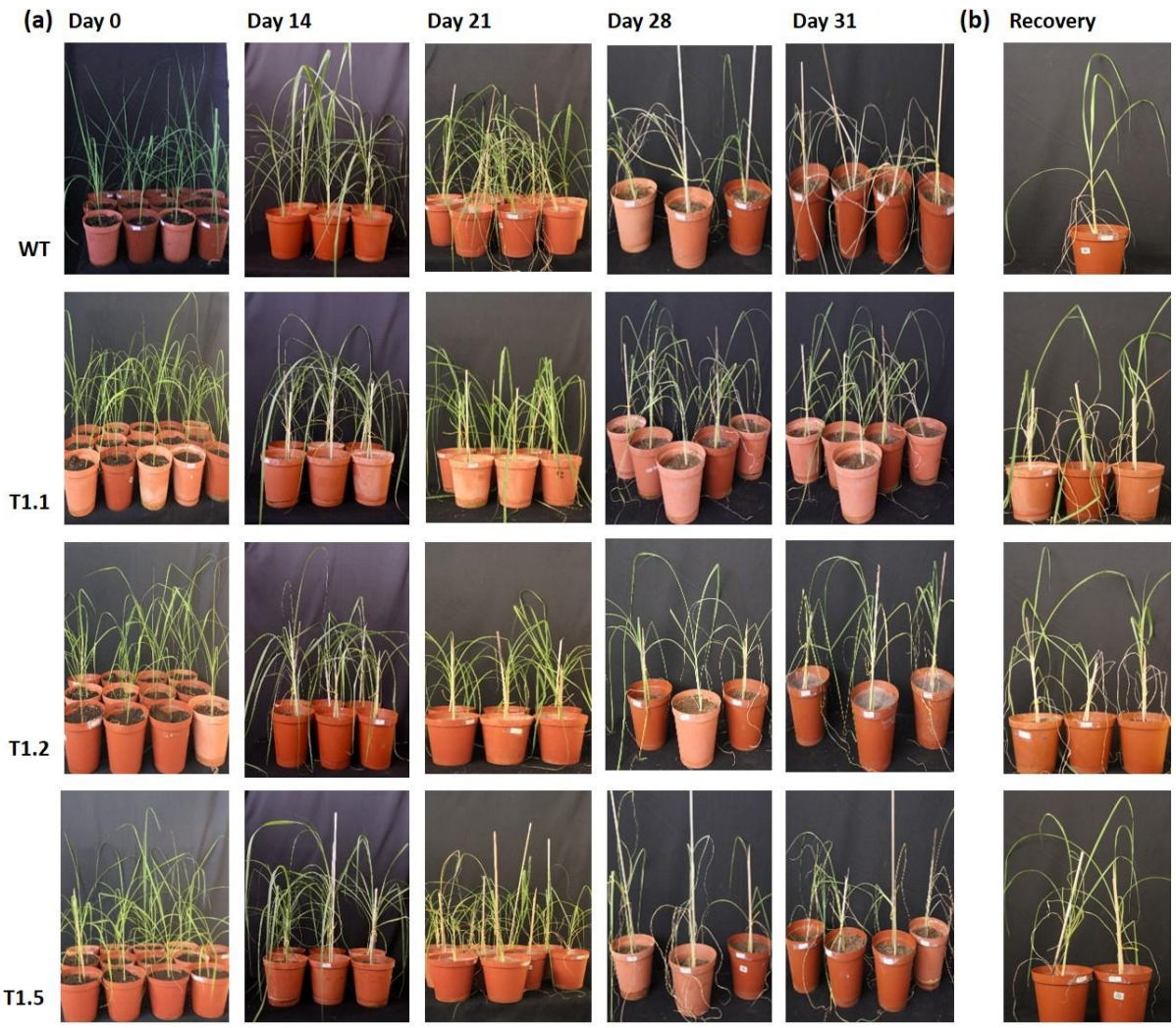

Figure 3.6. Monitoring the phenotypic responses of *SINAC2* transgenic sugarcane (lines T1.1, T1.2 and T1.5) and WT plants to water-deficit stress. **(a)** Water was withheld from plants for a period of 31 days after which the plants were **(b)** re-watered and the phenotypic recovery monitored after 14 days.

Table 3.3 Leaf width, length and shoot height of *SINAC2* transgenic sugarcane and WT plants evaluated every 7 days for a 21 days water-deficit period. Values present the average means ± standard deviation (SD) of six biological replicates (n = 6). (*) and (***) indicates significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.001$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test.

Plant line	Average leaf width (cm)	Average leaf length (cm)	Average plant height (cm)
Day 0			
WT	3 ± 0.3	66 ± 5.3	18 ± 1.9
T1.1	2 ± 0.3	64 ± 6	20 ± 3
T1.2	2 ± 0.3	56 ± 5.7	16 ± 1.5
T1.5	3 ± 0.4	73 ± 7.6	19 ± 3.4
Day 7			
WT	3 ± 0.2	70 ± 5.9	20 ± 2.3
T1.1	2 ± 0.2*	67 ± 5.5	20 ± 2.2
T1.2	2 ± 0.3	63 ± 7.7	17 ± 1.9
T1.5	3 ± 0.2	75 ± 3.9	20 ± 3.7
Day 14			
WT	3 ± 0	81 ± 6.4	21 ± 1.8
T1.1	2 ± 0.	78 ± 8.4	22 ± 2.7
T1.2	2 ± 0.3	68 ± 15	19 ± 1.6
T1.5	3 ± 0.2*	76 ± 4.5	20 ± 3.1
Day 21			
WT	3 ± 0.4	82 ± 5.3	23 ± 1.9
T1.1	2 ± 0.4***	79 ± 7.3	22 ± 3.1
T1.2	2 ± 0.4***	68 ± 15	21 ± 4.2
T1.5	3 ± 0.3	78 ± 4.2	22 ± 2.2

Table 3.4. Survival rates of *SINAC2* transgenic sugarcane (lines T1.1, T1.2 and T1.5) and WT plants exposed to 35 days of drought. For each genotype a total of 25 plants were included in the pot trial. The plants were assessed for damage after 25 days without water (ww) and again every second or third day after this time point. Plants were recorded as healthy when less than 5% of the leaves showed signs of browning. Values represent the number of plants being dead, damaged or healthy at each time point, expressed as a percentage.

Plant line	Dead (%)	Damaged (%)	Heathy (%)
Day 25			
WT	17	17	66
T1.1			100
T1.2		8	92
T1.5		17	83
Day 28			
WT	37	13	50
T1.1		8	92
T1.2		10	90
T1.5	12	12	76
Day 31			
WT	43	43	14
T1.1		22	78
T1.2		33	67
T1.5	29	29	42
Day 33			
WT	60	40	
T1.1	11	67	22
T1.2		33	67
T1.5	20	60	20
Day 35			
WT	75	25	
T1.1	25	62	13
T1.2		50	50
T1.5	50	50	

The level of water loss was expressed as the relative water content (RWC) recorded in the TVD leaf collected from the different transgenic genotypes and WT plants, which in turn coincides with the analysis of soil moisture content (SMC) (Figure 3.7). Results showed that the soil in all pots included in the trial were fully saturated at a level of ± 0.35 m^3/m^3 and the RWC in all plants was around 90% prior to withholding water (day 0 ww). Thereafter, the soil moisture content in all pots included in the trial gradually decreased at similar rates to an average value of 0.074 m^3/m^3 by day 28 ww (Figure 3.7a). During the course of the

applied water-deficit stress, wild type plants lost around 70% of their RWC by 28 day ww. Two transgenic lines, T1.1 and T1.2 had significantly higher RWC compared to WT plants at day 28 ww (Figure 3.7b).

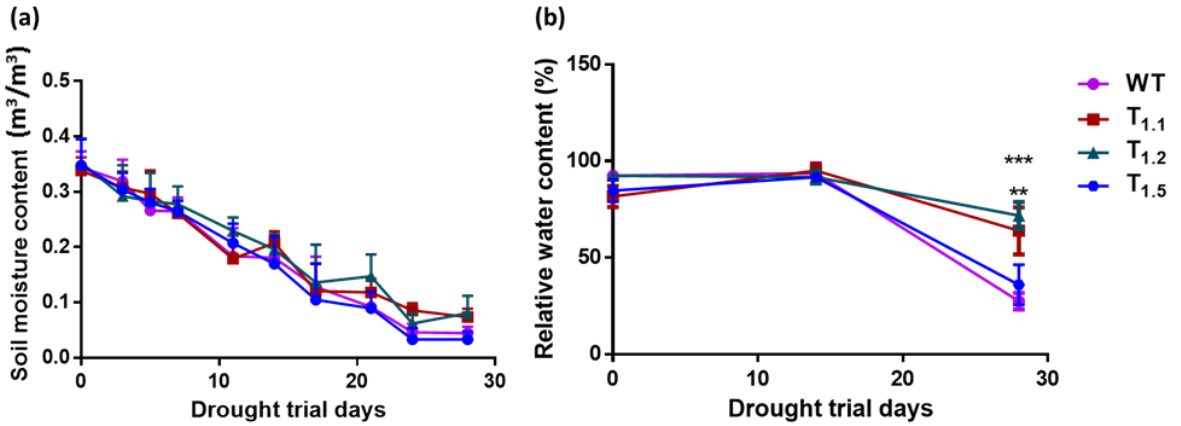

Figure 3.7 Comparative analysis of **(a)** soil moisture content of all pots and **(b)** relative water content (%) in the leaves of transgenic sugarcane (lines T1.1, T1.2 and T1.5) and wild-type (WT**)** plants prior to and after induction of water-deficit stress. Data is presented as means ± SD of three biological replicates (n = 3). (**) and (***) indicates significant differences compared to the WT at $p \leq 0.01$ and $p \leq 0.001$, respectively. Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test.

3.3.5 Root morphology of transgenic and WT sugarcane exposed to drought stress

The root system of the plants from the different genotypes was evaluated prior to and after 31 days without water. Due to the destructive nature of the evaluation, measurements were taken from a different sub set of three plants from each genotype at each time point. Results displayed a notable difference in the morphology and biomass of roots between transgenic and WT plants as observed at the start of the water-deficit stress (day 0 ww) (Figure 3.8 and Table 3.5). The roots system of the WT was bigger than the transgenic plants. Transgenic plants had an average root fresh weight between 3.8-4.2 g and dry weight of 0.5-0.6 g, which was significantly lower than WT, while WT plants recorded higher biomass values, 4.3 and 0.8 g of fresh and dry weight (Table 3.5), respectively.

Prior to initiation of water stress (day 0 ww), the roots of all plants were bulky with similar fresh weights, but significantly lower dry weights, recorded in WT and all the transgenic lines. However under extended water-deficit conditions (day 31 ww) the roots of transgenic plants seemingly grew longer lateral roots than WT plants. In all the genotypes the root biomass increase over the course of the stress period, the WT plants (3.4 g) more than the transgenics (1-1.5 g).

Figure 3.8 Comparison of the root growth of *SlNAC2* transgenic sugarcane (lines T1.1, T1.2 and T1.5) and WT plants prior to and after 31 days without water.

Table 3.5. Root biomass of *SINAC2* transgenic sugarcane (lines T1.1, T1.2 and T1.5) and WT plants measured during the 31 days of water-deficit period. Values present the average means ± standard deviation (SD) of three biological replicates (n=3). Statistical significance was determined using one-way ANOVA and variance using Bonferroni's multiple comparison test. (**) and (***) indicates significant differences compared to the WT at $p \leq 0.01$ and $p \leq 0.001$, respectively.

	WT	T1.1	T1.2	T1.5
DAY 0				
FW	4.3 ± 0.3	4.0 ± 0.4	3.8 ± 0.2	4.2 ± 0.3
DW	0.8 ± 0.02	0.5 ± 0.03**	0.5 ± 0.04***	0.6 ± 0.01**
DAY 28				
FW	4.3 ± 0.2	4.1 ± 0.4	3.9 ± 0.6	4.2 ± 0.2
DW	2.0 ± 0.2	1.3 ± 0.2	1.9 ± 0.1	1.8 ± 0.2
DAY 31				
FW	7.7 ± 0.5	5.0 ± 0.6	5.3 ± 0.7	5.5 ± 0.6
DW	1.5 ± 0.2	0.8 ± 0.01	0.9 ± 0.1	1.3 ± 0.2

3.3.6 Photosynthetic activity in transgenic and WT sugarcane under drought stress

The role of *SINAC2* on photosynthetic activity, which in turn is influenced by stomatal conductance, was investigated in the different genotypes during the 31 days of drought stress. The photosynthetic activity (Fv/Fm) in all genotypes prior to water-deficit stress was at the same level with chlorophyll fluorescence values closed to 0.75. Over the course of the stress period, chlorophyll fluorescence decrease only slightly in all lines. Measurements were always taken from a subset of plants within the healthy and/or damage classification at the different time points. For obvious reasons measurements could not be taken from dead plants of which the leaves were dry and brittle and where chlorophyll fluorescence would have dropped significantly and ceased completely. After 31 days ww, all surviving transgenic plants from the different lines maintained a higher photosynthetic rate, with lines T1.1 and T1.2, at significantly higher levels than WT plants (Figure 3.9 a).

The initial stomatal conductance in the different plant genotypes ranged between 180 and 250 mmol/m^2s at day 0 ww and increase up to day 7 ww. At this stage only transgenic line T1.1 had significantly lower stomatal conductance levels than the WT plants (Figure 3.9b).

After this time point the extension of water-deficit stress caused a similar decline in the stomatal conductance of all plants. Throughout the rest of the drought stress even under severe (day 31 ww) stress conditions, most transgenics had high levels of stomatal conductance with line T1.1 significantly higher than WT plants (Figure 3.9 b).

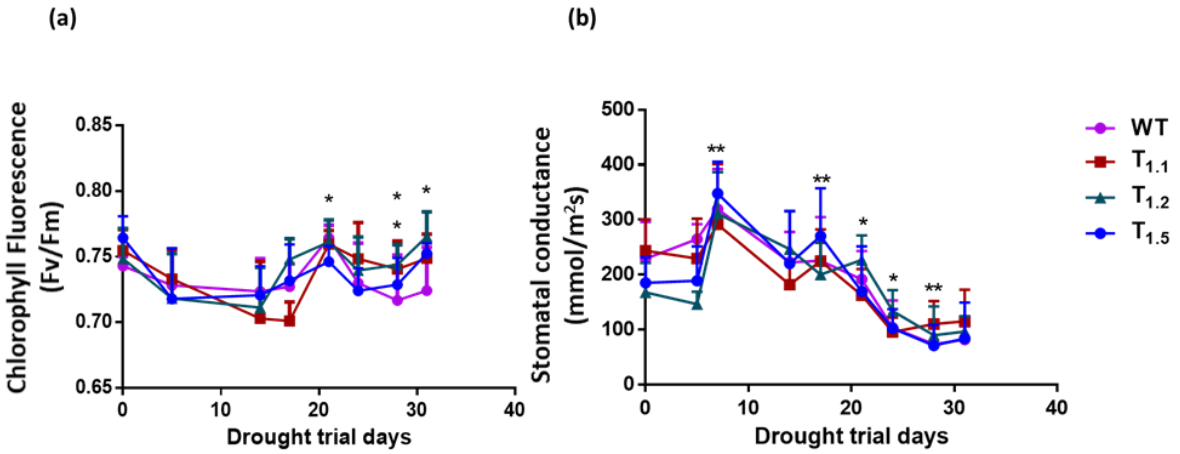

Figure 3.9. Analysis of photosynthetic machinery of transgenic sugarcane (lines T1.1, T1.2 and T1.5) and wild-type (WT) plants prior to (day 0) and after exposure to water-deficit stress. Measurements of **(a)** chlorophyll fluorescence (Fv/Fm) and; **(b)** stomatal conductance are presented as the mean ± SD of six biological replicates (n = 6). (*) and (**) indicates significant differences compared to the WT at $p \leq 0.05$ and $p \leq 0.001$, respectively. Statistical significance was determined using one-way ANOVA and varience using Bonferroni's multiple comparison test.

3.3.7 Subcellular localization

3.3.7.1 Cloning and Agrobacterium transformation of SINAC2

To determine the subcellular localization of *SINAC2*, the gene was cloned into the pENTR™/D-TOPO donor vector provided by the pENTR™ Directional TOPO Kit. The full length *SINAC2* gene was successfully obtained through PCR using pJET1.2:*SINAC2* as template and gene specific primers, which was designed to add an additional CACC sequence for cloning purposes (Figure 3.10). The 964 bp amplicon was subsequently cloned inside the *attL* sites in the donor vector and the recombinant plasmid was then transformed into competent OneShot®Top10 *E. coli* cells. A combination of transgene and vector specific

primers were used to confirm successful cloning and transformation into *E. coli* colonies (Figure 3.11).

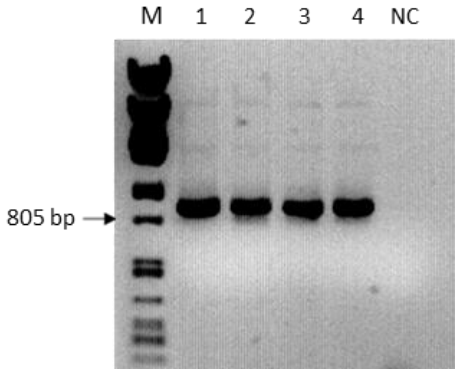

Figure 3.10. PCR analysis confirming the full length *SlNAC2* entry into the pENTRY vector (lanes 1-4; amplicon size 964 bp) into OneShot®Top10 *E. coli* cells. M (*Pst*I λ marker); and NC (negative H_2O control).

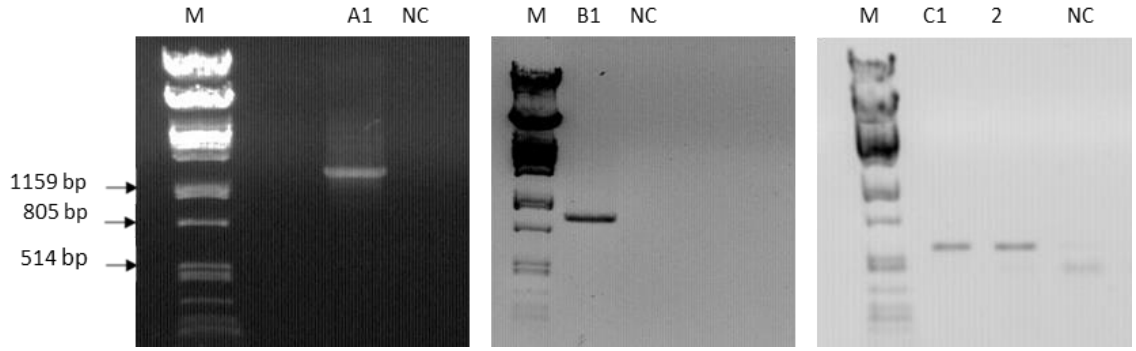

Figure 3.11. PCR analysis confirming cloning and transformation of pENTRY:*SlNAC2* into OneShot®Top10 *E. coli* cells, where clones were tested with M13 vector specific (A; amplicon size 1305 bp); ENTRY cloning (B; amplicon size 946 bp); and two clones with *SlNAC* specific (C; amplicon size 600 bp) primers sets. M (*Pst*I λ marker); and NC (negative H_2O control).

The pENTRY:*SlNAC2* vector was sub-cloned into the pGWB506 destination vector containing the enhanced green fluorescence protein (*EGFP*), and the full length *SlNAC2* sequence was cloned downstream of the *EGFP* reporter creating a fusion cassette, *EGFP*:*SlNAC2.* The fusion cassette was placed under the control of a cauliflower mosaic virus (CaMV) 35S promoter and *nopaline synthase* (*nos*) terminator (Supplementary data, Figure S8). The destination vector (pGWB506*:SlNAC2*) was then transformed into competent OmniMAX *E.*

coli cells (Figure 3.12, lanes 1-3) and subsequently into *Agrobacterium tumefaciens* LBA4404 cells via electroporation (Figure 3.12, lanes 4-6).

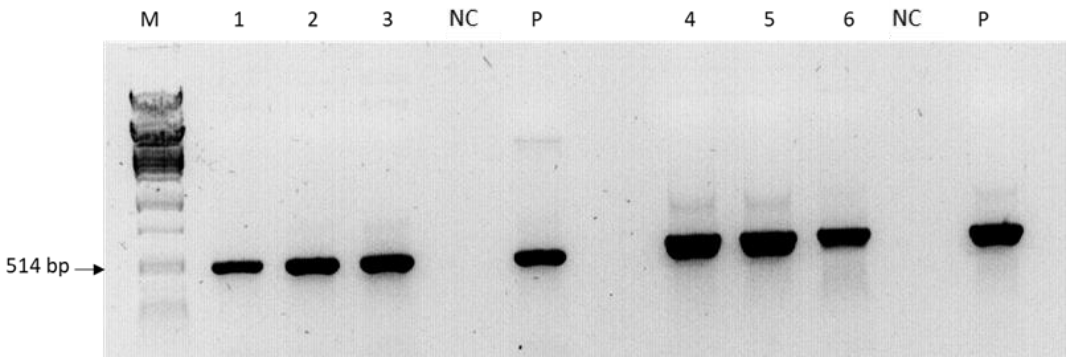

Figure 3.12. Colony PCR analysis confirming sub-cloning of *SlNAC2* into the pGWB506 destination vector and transformation into OmniMAX *E. coli* cells (lanes 1-3) and *A. tumefaciens* (lanes 4-6). M (*Pst*I λ marker); and NC (negative H_2O control); P (positive plasmid control; pJet1.2:*SlNAC2*).

3.3.7.2 Histology analysis of transient gene expression

The two vector constructs, pGWB506:*SlNAC2* and pGWB506 (control) were transfected independently into onion epidermis cells via *Agrobacterium*-mediated infiltration. The transiently expression of the reporter gene were viewed 48, 60 and 72 hours after incubation in the dark. Fluorescence was seen in the cells transformed with the recombinant vector construct after 72 hours (Figure 3.13). In the recombinant vector image, weak green fluorescent spots were observed in the microscope reflection field, which when cross linked with bright field images indicated fluorescence signals potentially localized to the nucleus and cell membrane. However, due to poor fluorescent signals and manual overlaying with bright field images the exact localization of the *GFP:SlNAC2* signal could not be absolutely determined and as a result, this experiment will have to be repeated.

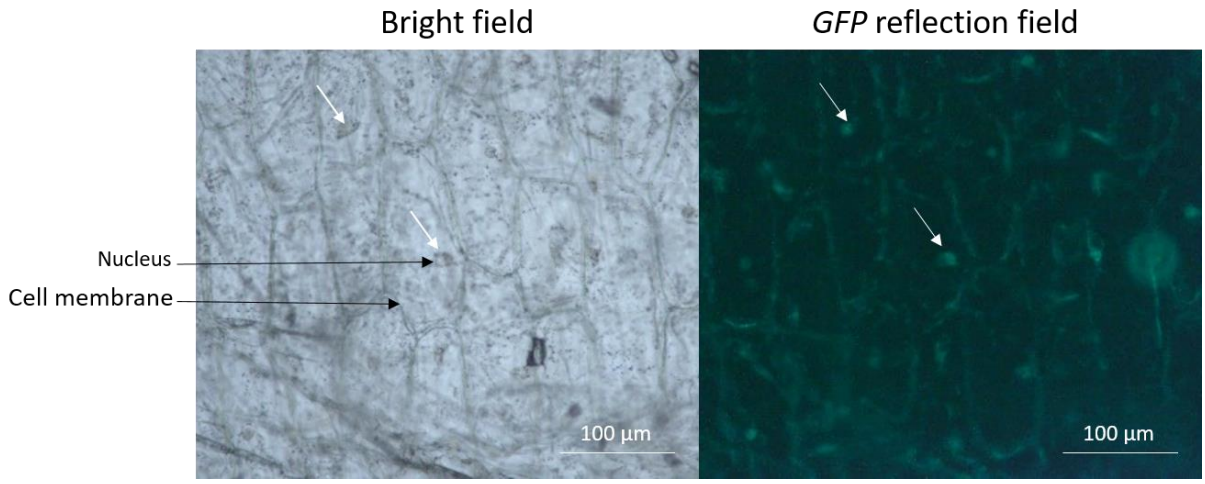

Figure 3.13. Histology analysis of the transient gene expression of *GFP* to determine the subcellular localization of the *SlNAC2* gene in onion epidermis cells transformed with the pGWB506:*SlNAC2* recombinant vector construct. Images were visualized on an A1 microscope connected to ZEISS Axiocam 305 color imaging software at 10x magnification.

3.4. DISCUSSION

Plant based NAC transcription factors take part in the activation of genes linked to water-deficit stress responses (Sakuraba *et al.,* 2015a; Wang *et al.,* 2016c). These TF perceive stress signals through either ABA-dependent or -independent pathways. Some studies discovered that plant response to stress, controlled by a NAC protein, may vary in species and although several studies have investigated the NAC function in plants, little is known about the functions of NAC genes in sugarcane. In this study we started to elucidate the function of a tomato NAC TF in transgenic sugarcane plants exposed to drought. Recent overexpression of this *SlNAC2* gene from tomato improved tolerance to drought in transgenic tobacco and osmotic stress tolerance in *Arabidopsis* plants. The gene was shown to regulate expression of stress responsive genes, which take part in the regulation of osmo-protectant accumulation and antioxidant genes responsible for reactive oxygen species detoxification (Borgohain *et al.,* 2019; van Beek, 2018). *SlNAC2* transgenic tobacco plants also recovered better from the stress compared to WT plants after exposure to severe drought stress (van Beek, 2018).

In this study, the same *SINAC2* gene, which was received from our collaborator Prof SK Panda (India), was cloned into the pUBI510 plant expression vector (Figure 3.1 and 3.2) and further transformed into sugarcane calli to generate *in vitro* transgenic plantlets using particle bombardment and somatic embryogenesis. PCR analysis confirmed five clones to contain and express the *SINAC2* transgene with only transgenic line T1.3 showing relatively low *SINAC2* transcript levels compared to the T1.1, T1.2, T1.4 and T1.5 genotypes (Figure 3.5). Overexpression of *SINAC2* in transgenic sugarcane lines, T1.1, T1.2 and T1.5 enhanced drought tolerance in preliminary greenhouse pot trials, where plants were exposed to 21 days (mild) and 35 days (severe) water-deficit stress conditions.

During these trials the soil moisture in all the pots included in the trial decreased to dry, from a fully saturated (average 0.35 m^3/m^3) state to less than 0.074 m^3/m^3 over 28 days of water-deficit stress (Figure 3.7 a). During this water-deficit period, a high loss in water content was detected in the WT sugarcane plants. Most transgenic plants retained a RWC significantly higher than WT plants (Figure 3.7b), consistent with the possibility that overexpression of *SlNAC2* led to osmotic adjustment and displayed tolerance to drought under extreme dehydration. According to Borgohain *et al*. (2019), *SINAC2* transgenic *Arabidopsis* plants also maintained a higher RWC under osmotic stress.

In terms of morphological differences between the genotypes during exposure to drought, the transgenic sugarcane displayed mostly similar growth patterns as the WT plants. Only the leaf width of the transgenic plants were significantly less than the WT plants after 21 days of water-deficit stress (Figure 3.6, Table 3.3). Also, WT plants started to senesce earlier than the transgenic plants during the stress imposed period (Table 3.4). Transgenic plants displayed a delay in senescence by retaining fully green leaves, while the WT plants started to exhibit leaf wilting, drying and yellowing (chlorosis). In sugarcane, some of the most common and early morphological drought responses included leaf senescence and the reduction in leaf area (Inman-Bamber *et al*., 2012). In dehydration tolerant sugarcane varieties, stay-green phenotypes are considered valuable morphological traits in sustaining yield (Wagih *et al.,* 2003). NAC proteins have been shown to play vital roles in regulating leaf senescence in several plant species. In combination with environmental conditions, NAC TFs reprogram the onset, development and conclusion of leaf senescence (Kim *et al*., 2016).

Senescence is known to be the last stage of leaf development and is an active, genetically regulated process driven by TFs, triggered by aging signals and environmental circumstances (Li *et al*., 2018). Dozens of NAC TFs are widely known to accelerate this process and transciptome profiling has indentified large numbers of NAC TF genes upregulated specifically, in *Arabidopsis* 30 NAC genes, during stress-induced leaf senescence (Breeze *et al*., 2011). Some examples include *AtNAC080, ANAC029 (*NAC-LIKE, ACTIVATED by APETALA3/PISTILLATA), *OsNAC2* and *OsNAP.* These genes, when suppressed in transgenic rice and *Arabidopsis* delayed leaf senescence or when overexpressed enhanced senescence in the transgenic plants (Guo and Gan, 2006; Li *et al*., 2018; Liang *et al.,* 2014; Sakuraba *et al.,* 2015b; Mao *et al*., 2017). Furthermore, NAC TFs such as *ANAC092 and AtNAC2* have been shown to regulate the expression of *SENESCENCE ASSOCIATED GENES* (*SAGs*) (Balazadeh *et al.,* 2010; Kim *et al.,* 2009). *SENESCENCE ASSOCIATED GENES* are known to be involved in the regulation of processes associated with senescence (Sarwat and Tuteja, 2018). *TaNAC29* specifically regulates ABA signalling and reduced expression of *RD29A, SAG13, SAG113, AIB1, ERD11*, and *AB15* genes (Gahlaut *et al.,* 2016; Huang *et al.,* 2015). In contrast NAC TF can also delay leaf senescence, as was the case with *DRL1*, a NAC TF isolated from grapevine, when overexpressed in tobacco significantly delayed leaf senescence (Zhu *et al*., 2019). In wheat, *TaNAC-S* delayed leaf senescence in transgenic plants (Zhao *et al*., 2015). VNI2 (VASCULAR-RELATED NAC-DOMAIN INTERACTIN2; (also known as *ANAC083*) overexpression strongly functioned by delaying senescence through interactions with ABA (Finkelstein, 2013; Wu *et al*., 2012; Yang *et al*., 2011). When *SlNAC2* was overexpressed in *Arabidopsi*s and tobacco, the NAC protein protected the transgenic plants against precocious leaf senescence by maintaining chlorophyll content (van Beek, 2018; Borgohain *et al*., 2019). In the *SlNAC2* transgenic sugarcane, the perseverance of the green leaf phenotype most likely also ensured continued photosynthetic activity in the leaves of the transgenic plants.

Under water stress, plant cells dehydrate and are forced to amend their cell properties to conserve water. In response to drought, ABA is synthesized and signal stomatal closure to contain water loss through transpiration, thus prevent cellular dehydration (Schroeder *et al.,* 2001). The involvement of NAC proteins in stomatal closure and opening remains to a large extent unclear (Cominelli *et al.,* 2010; Negi *et al.,* 2018). However, *SNAC1* and *SNAC2*

are known to participate in regulating stomatal movement via ABA signalling in rice. Transgenic rice overexpressing these genes had increased tolerance to severe dehydration through reduced transpiration due to a high stomatal closure in the guard cells, compensated by enhanced root growth, without affecting plant yield (Hu *et al.,* 2006; Joshi *et al*., 2016; Singh *et al.,* 2015). In transgenic cotton plants, overexpressing *SNAC1*, transpiration decrease during the reproductive phase but not the flowering stage, furthermore ensuring yield maintenance (Liu *et al*., 2014a). Although stomatal closure is a significant physiological response for preventing water loss it is also known to cause a decrease in the rate of photosynthesis (Lopes *et al.,* 2011; Marchiori *et al.,* 2017; Zlatev and Yordanov, 2004).

The results obtained in this study showed that overexpression of *SINAC2* in most transgenic sugarcane plants resulted in significantly higher rates of quantum yield of photosystem II (Fv/Fm) than WT plants when water-deficit stress were extended past 21 days ww, during the same period the transgenic plants also maintained significantly higher levels of stomatal conductance (Figure 3.9). Maintenance of photosynthetic rate and stomatal conductance are considered important traits used to differentiate between drought susceptible and resistant sugarcane genotypes (Ferreira *et al.,* 2017). Also, these physiological parameters linked to drought tolerance are known to allow plants to easier repair cellular damage after the stress period (Liberato *et al.,* 2006; Souza *et al.,* 2004). *SINAC2* overexpression in the sugarcane increased the recovery rate of most transgenic plants compared to the WT plants (Figure 3.6).

Root architecture are also useful to forecast the ability of plants to adapt to water-deficit stress. In sugarcane the development of deep and large root structures are used as selection criteria for genotypes with enhanced water stress tolerance (Smith *et al*., 2005). While drought inhibit shoot growth the roots may continue to elongate and in sugarcane, this is an adaptive mechanism that facilitates water uptake (Bartels and Sunkar, 2005; Munns *et al*., 2000). In this study, preliminary assessment of root characteristics prior to and after dehydration stress in the different sugarcane genotypes indicated potentially different root growth patterns. Root dry mass prior to stress exposure was significantly lower in the transgenic plants compared to the WT plants (Table 3.5). NAC TFs are known to influence root growth both limiting and enhancing biomass and altering root architecture under

drought conditions but also when consitutively overexpressed in transgenic plants under non-stress conditions (Quach *et al*., 2014; Yang *et al*., 2019). For example, the *OsNAC9* TF gene when constitively overexpressed in rice, altered the plants' root architecture under field conditions (Redillas *et al*., 2012). As expected, root biomass increased during the stress period in all genotypes (Figure 3.8 and Table 3.5). However, at the end of the stress period, the root systems of the *SlNAC2* transgenic plants tended to be longer and less bulky. Similar results were obtained by Bidoia *et al*. (2006) and Queiroz *et al*. (2011) when sugarcane cultivars developed long and thin roots under conditions of water-deficiency. Increase root length is considered a desirable trait in sugarcane to extract deep soil moisture when water is limited and are found in water stress tolerant sugarcane genotypes (Endres *et al*., 2010; Thiebaut *et al*., 2014). Upon water-stress detection, the roots expand and elongate into soil layers containing water for absorption. This then prevent dehydration in plants under limited water conditions (Glover, 1967; Namwongsa *et al.,* 2018; Smith *et al.,* 2005).

NAC TFs are known to influence root architecture under dry conditions. Lateral root growth was enhanced in transgenic *Arabidopsis* and rice overexpressing *TaNAC2* and radial root growth upon *OsNAC5*, *OsNAC9* and *OsNAC10* overexpression, all of which resulted in enhanced drought tolerance (Jeong *et al.,* 2010, 2013; Redillas *et al.,* 2012). Furthermore, transgenic plants overexpressing *AtNAC2* (He *et al.,* 2005), *NAC1* (Xie *et al.,* 2000), *GmNAC11, GmNAC20* and *GmNAC109* (Hao *et al.,* 2011; Yang *et al.,* 2019) enhanced lateral root development through auxin signalling pathways in drought, salt and freezing conditions. According to a study by Wang *et al.* (2016b), *SlNAC35* overexpression in tobacco also enhanced lateral and longer root length and development through expression of *auxin responsive factor (ARF)* genes. Studies showed that *OsNAC10* known to be specifically expressed in the roots, regulated by a root promoter, contribute to drought tolerance by enhancing root growth and development in transgenic rice, *Arabidopsis* and cotton plants under water-deficit stress (Gunapati *et al.,* 2016; Joeng *et al.,* 2010; Lee *et al.,* 2017). Therefore, the established link between NAC TFs and root growth, as reported in literature, and preliminary results collected in this study, suggest that *SlNAC2* overexpression promote deep root growth, which might contribute to the observed enhanced drought stress tolerance in the transgenic sugarcane plants.

A recent review by Mohanta *et al.* (2020) suggested that the diversity of NAC TF's functional roles in plants may indicate localization in various organelles, including the cytosol and plasma membrane. Studies confirm that the majority of NAC proteins contain a signalling sequence for a nuclear localization and few are membrane bound associated with the endoplasmic reticulum (ER) (Fang *et al.,* 2015; Zhang *et al.,* 2020). A subcellular localization study conducted by Yang *et al.* (2014) found that the *SINAC2* protein, encoded by a gene isolated from *Suaeda liaotungensis*, is localized in the nucleus of the cell. In this study, we investigated the subcellular localization of *SINAC2*. *Agrobacterium*-mediated infiltration liquid was used to transfer the enhanced green fluorescent protein (*EGFP*), fused with *SINAC2* in the pGWB506 destination vector, into onion epidermis cells. Microscope reflection showed *EGFP* signals possibly localized in the nucleus and cell membrane of the plant cell (Figure 3.13). However, due to weak fluorescence and microscopic imaging, we could not confirm *SINAC2* localization in the nucleus and the experiment will have to be repeated.

CONCLUSIONS AND FUTURE PROSPECTS

Drought is a global, recurring characteristic feature of current extreme weather patterns. It affect sustainable agriculture and has socio-economic consequences in especially rain-fed regions. South Africa has also been experiencing unpredictable and frequent drought episodes over the last few years, causing agricultural crop losses in certain production areas (Jones and Singels, 2015; Vetter, 2009). While this is a concern, water supply and quality is still deteriorating and thus, impact crop yield and food security negatively. Drought also directly affect agricultural economics in terms of food prices, decreased levels of employment, income, and also nutrition and health (Dube and Jury, 2003; Hussain *et al.*, 2008; Nhamo *et al.,* 2019).

For sugarcane, its growth and productivity is critically constricted by water-deficit (Lakshmanan and Robinsosn, 2014). Drought causes damage during developmental processes of sugarcane, with tillering and stem elongation being the most sensitive phases. Drought is known to affect sugarcane by decreasing stomatal conductance and reducing the photosynthetic rate, mainly due to stomatal limitations. This along with impairment in the stalk and leaf growth, reduce leaf area, causing leaf rolling and senescence, which in turn result in crop losses and decrease sugar quality and biomass (Ferreira *et al.,* 2017; Khaled *et al.,* 2018). To sustain this crop, developing cultivars with improved tolerance towards drought is extremely important. Crop improvement using genetic engineering to manipulate regulatory genes, which in turn can act as molecular switches to activate stress-responsive genes, can potentially protect plants against the negative effects of drought.

Transcription factors act as important switches of transcription networks and amongst others control expression of genes related to the abiotic stress responses in plants. The underlining aim of my MSc project was to determine whether overexpression of two specific TFs can enhance the ability of sugarcane to withstand drought without the constitutive overexpression of the transgenes affecting plant growth and the accumulation of sucrose and other carbohydrates. The hypothesis is that the overexpression of exogenous TFs can lead to improved physiological and biochemical performance in the transgenic plants leading to enhance stress tolerance. Our current knowledge of the regulatory functions of

stress-responsive TFs in various plant species indicate potential use of these genes as candidates for generation of stress tolerant transgenic plants under adverse conditions. Furthermore, overexpressing TFs in sugarcane can be a useful biotechnology tool in investigating the function of these TFs in a commercially important crop species.

As part of the first set of objectives outlined for my MSc research project, I have successfully cloned two TFs, one isolated from *Arabidopsis*, a B-box TF, and one isolated from tomato, a NAC TF, in an appropriate plant expression vector. I have then proceeded and genetically transformed sugarcane with these transgenes independently and confirmed as part of objective 3, the successful insertion and expression of the transgenes in sugarcane plants. I have then continued to assess the transgenic plants for their capacity to tolerate drought.

The first part of my study focused on a B-box TF gene isolated from *Arabidopsis*, namely *AtBBX29*. B-box transcription factors are part of the large zinc finger TF gene family. So far a limited number of studies have been conducted to determine the role of B-box TFs in abiotic stress responses in plants and to my knowledge none at all in sugarcane. These B-box TFs are known to transcriptionally activate or suppress downstream gene responses through protein binding to *cis*-elements in the promoter regions of DNA, facilitating RNA binding or forming protein to protein interactions. Studies have identified transcriptional targets associated with abiotic stress tolerance for C2H2 zinc finger proteins, which include B-box type TFs, to include genes related to ion balance, biosynthesis of substances for osmotic adjustment, antioxidant scavengers for reactive oxygen species (ROS) detoxification and genes involved in hormone signal transduction (Han *et al.* 2020; Liu *et al*. 2019).

In this study the overexpression of *AtBBX29* resulted in enhanced drought tolerance in the majority of transgenic sugarcane plants. This means the aim of my MSc research project, to generate drought tolerant sugarcane genotypes, was successfully accomplished and the data collected for this part of my research project was processed into a manuscript format and submitted for publication. In contrast the WT plants showed early signs of senescence and the majority of plants died before the transgenic plants.

Despite the water loss in the plants, *AtBBX29* overexpression ensured a high survival rate and helped maintain a healthy phenotype over an extended stress period in the transgenic plants. One of the transgenic lines, T1.8 seemed to be more sensitive towards drought with

a lower survival rate and low RWC after extended water-deficit stress compared to the other two transgenic lines. The high level of expression of the transgene or the insertion site of the transgene into the polyploid genome of this genotype might explain the variation in phenotype under drought. Data regarding the transgene copy number and quantitative transcript levels in the different transgenic genotypes need to be collected in the future.

As part of set objective 4, biochemical and physiological data was collected from the *AtBBX29* transgenic plants exposed to drought. Data indicated that the transgenic sugarcane plants retained higher photosynthetic performance throughout the drought period, due to adjustments in stomatal conductance and maintenance of the efficiency of photosystem II. The data collected during this study also showed that the transgenic sugarcane plants accumulated significantly higher levels of proline, which most likely contributed towards the osmotic adjustment in cells of the stressed plants (Hayat *et al.*, 2012). Moreover, drought tolerance in transgenic sugarcane plants was enhanced through an increase in the activivties of antioxidants, specifically superoxide dismutase (SOD) and catalase (CAT), which potentially could be linked with the observed decrease in the accumuation of H_2O_2 and MDA. However, SOD and CAT are not the only antioxidant enzymes that could have detoxified ROS. This study should be extended in the future to include the measurement of additional antioxidants, both non-enzymatic and enzymatic, such as ascorbate peroxidase (APX), an enzyme with a high affinity for H_2O_2 detoxification, to obtain a more complete picture of the role of *AtBBX29* in maintaining ROS homeostasis and redox signalling in transgenic sugarcane plants under drought stress. In a recent review conducted by Han *et al*. (2020), the role of C2H2 zinc finger proteins in maintaning stable ROS levels through directly targeting of antioxidase gene expression, linked with removal of ROS, was clearly shown (Gadjev *et al.,* 2006).

An additional outcome of the MSc study involved an extensive phenotypic characterization, as part of objective 5, to explore the impact of constitutive overexpression of a B-box TF on the development and growth of sugarcane plants. In the current study the *AtBBX29* gene was under control of the strong constitutive ubiquitin promoter. This promoter ensures the continued expression of the transgene in all tissue and organs. This method of expression has potential setbacks such as reduced plant growth and accummulation of biomass. Despite initial concerns that the constitutive overexpression of a B-box TF might influence

the growth of the transgenic sugarcane plants, no impact was seen under normal environmental conditions. Phenotypic analysis showed that carbohydrates, growth and development of transgenic sugarcane plants grown to maturity remained unchanged when compared to WT plants.

Further future aspects to extend this study and our knowledge of the regulatory role of *AtBBX29* may include an investigation into the complete downstream gene expression profile through sequencing of the transcriptome of the transgenic sugarcane exposed to drought under the regulation of *AtBBX29.* This will enhance our understanding in the working mechanism of B-box TF in the stress-response of sugarcane.

In the second part of my MSc research project, a member of the NAC (no apical meristem, ATAF1/2, and CUC2 [cup-shaped cotyledon]) family of transcription factors, one of the largest plant-specific TF families actively involved in plant fitness under stress, was overexpressed in sugarcane. A number of studies have proven that the manipulation of NAC TFs in transgenic plants confer stress tolerance to plants. However, little is known about the functions of NAC genes in sugarcane.

As part of objective 2 a NAC TF from tomato, *SlNAC2* was successfully introduced and overexpressed in sugarcane. Overexpression of this TF in sugarcane plants seem to enhance drought tolerance in the transgenic plants, as was indicated by the preliminary greenhouse pot trials that was conducted where plants were exposed to 21- 35 days of water-deficit stress. Increased tolerance in *SlNAC2* overexpressing sugarcane plants was associated with transgenic plants retaining higher RWC towards the end of the water-deficit stress period compared to WT plants. Transgenic plants displayed delayed senescence and increased survival rates. *SlNAC2* overexpression maintaned high photosynthetic activity, in terms of stomatal conductance and the quantum efficiency of photosystem II, in the transgenic plants. Furthermore, overexpression of *SlNAC2* seem to reduced root biomass in favour of enhancing the development of a deep rooting system, which might contribute to the observed enhanced drought stress tolerance in the transgenic sugarcane plants. NAC TFs are known to regulate downstream genes in auxin signalling crucial for deep rooting under stress conditions (He *et al.,* 2005; Xie *et al.,* 2000; Wang *et al.*, 2016).

Most transgenic plants also recovered better from the stress compared to WT plants, which might be due to the sustained maintenance of photosynthesis and stomatal conductance allowing a quick recovery once water becomes available again. However, a comprehensive investigation into the physiological and biochemical adjustments in the transgenic plants still need to be conducted. Due to time constraints, these experiments could not be introduced and concluded during the course of this MSc degree, and data for this is still outstanding. Physiological and biochemical data such as oxidative damage, anti-oxidative response and biosynthesis of osmotic adjustment substances need to be conducted to extend analysis of the transgenic plants in future studies.

According to previous studies, NAC proteins can regulate the components of ABA receptors (RCAR) involved in ABA signalling (Joshi-Saha *et al.,* 2011) and enhances expression of stress responsive genes (*rd29A/B, rd22* and *ERD1)* for osmotic tolerance and senescence. Therefore, investigating ABA involvement and relevant signalling gene expression profiles would be worthwhile in extending our knowledge of NAC control in sugarcane exposed to abiotic stress. Furthermore, NAC TFs also enhances dehydration stress tolerance through interactions with other TFs, such as WRKY, MYB and MYC via ABA-dependent signalling transduction pathways, which might be a further avenue to investigate in the transgenic sugarcane plants (Finkelstein *et al.,* 2002; Shinozaki *et al.,* 2003). This study also did not look at the effect of constitutive overexpressing *SlNAC2* on growth and sucrose yield in the transgenic plants. Occasionally, the overexpression of NAC TFs in transgenic plants resulted in negative effects on plants such as lower yields and stunted growth (Kato *et al.,* 2010; Nakashima *et al.,* 2007), if this turn out to be the case in the transgenic sugarcane plants, the overexpression of *SlNAC2* under control of a stress-inducible promoter will have to be considered.

Ultimately, it is also recommended that all transgenic plants overexpression the two TFs will have to be evaluated in field trials, both under stressed and non-stress environments. Glasshouse conditions are not a true representation of field conditions and performances of potted plants most likely differ from plant performance under field conditions.

Understanding the molecular mechanisms of TFs networks in crop plants exposed to abiotic stress will be essential for the development of stress tolerance crops that can better cope with drier environments in future climates.

SUPPLEMENTARY DATA

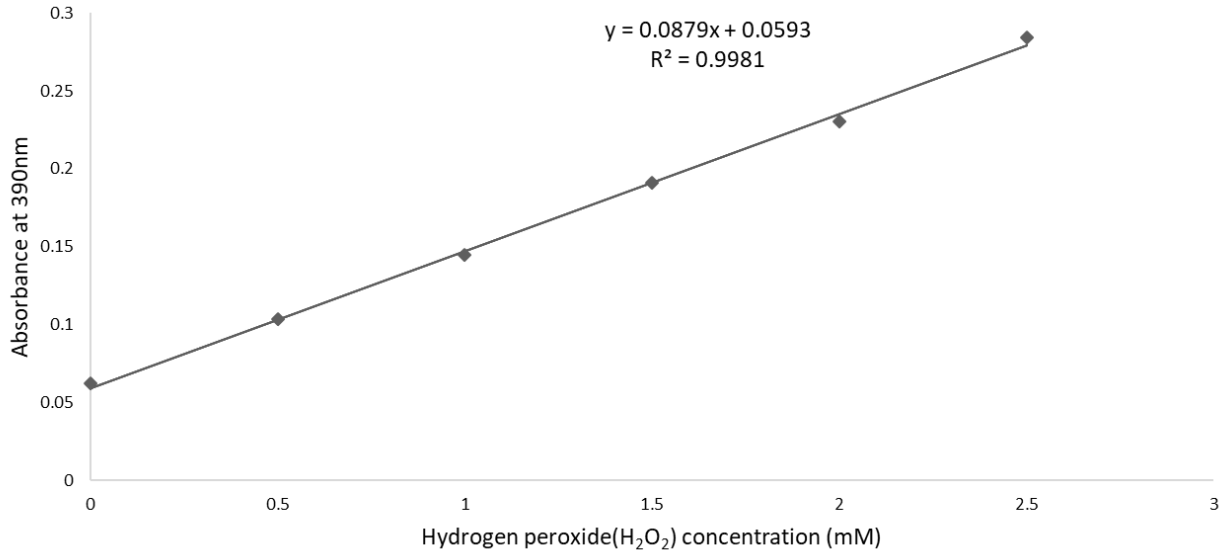

Figure S 1. Standard linear curve of hydrogen peroxide (H_2O_2) activity as described by Junglee *et al*. (2014). H_2O_2 concentrations ranged between 0 and 2.5 mM of 12% (m/v) H_2O_2 and the dilution series were setup in triplicate. Activity was calculated using the linear equation where x = H_2O_2 concentration and y = absorbance value.

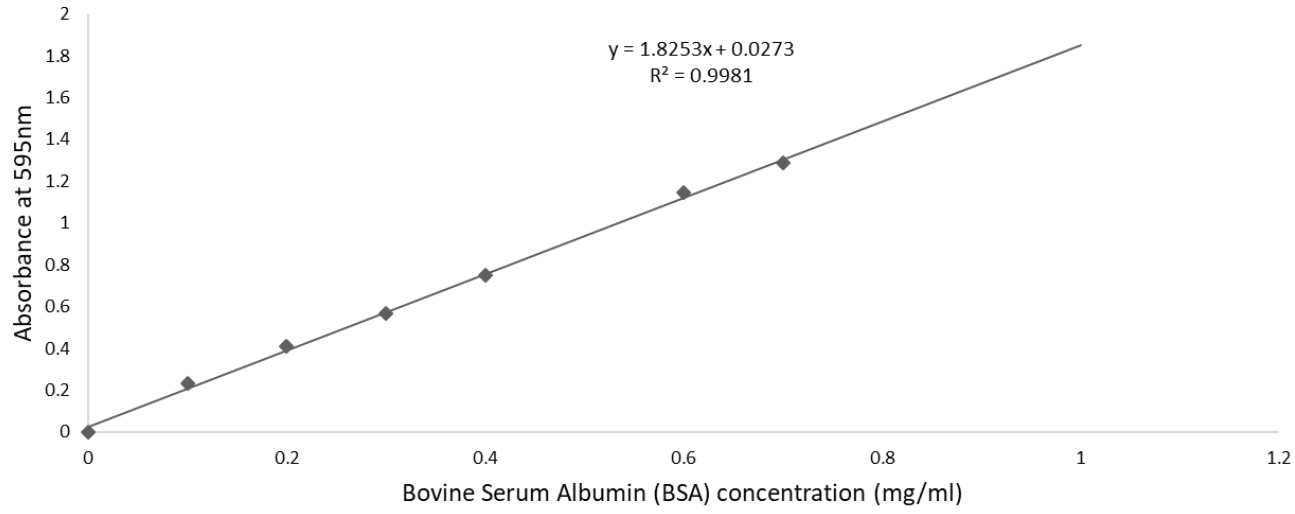

Figure S 2. Standard linear curve of bovine serum albumin (BSA) concentrations used in the determination of total protein content as described for the method of Bradford (1976). Protein concentration in homogenate was calculated using the linear equation where x = protein concentration and y = absorbance value.

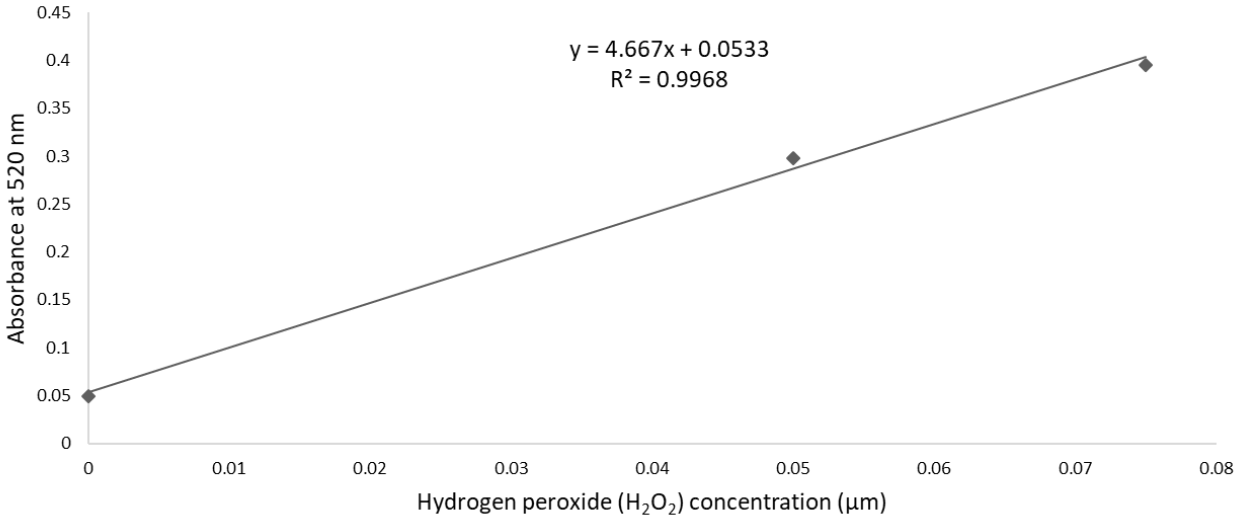

Figure S 3. Standard linear range of the catalase activity presented as the absorbance of red quinone imine dye at $OD_{520.}$ CAT concentration were calculated using the linear equation where x = protein concentration and y = absorbance value was used to calculate the amount of H_2O_2 after catalase activity.

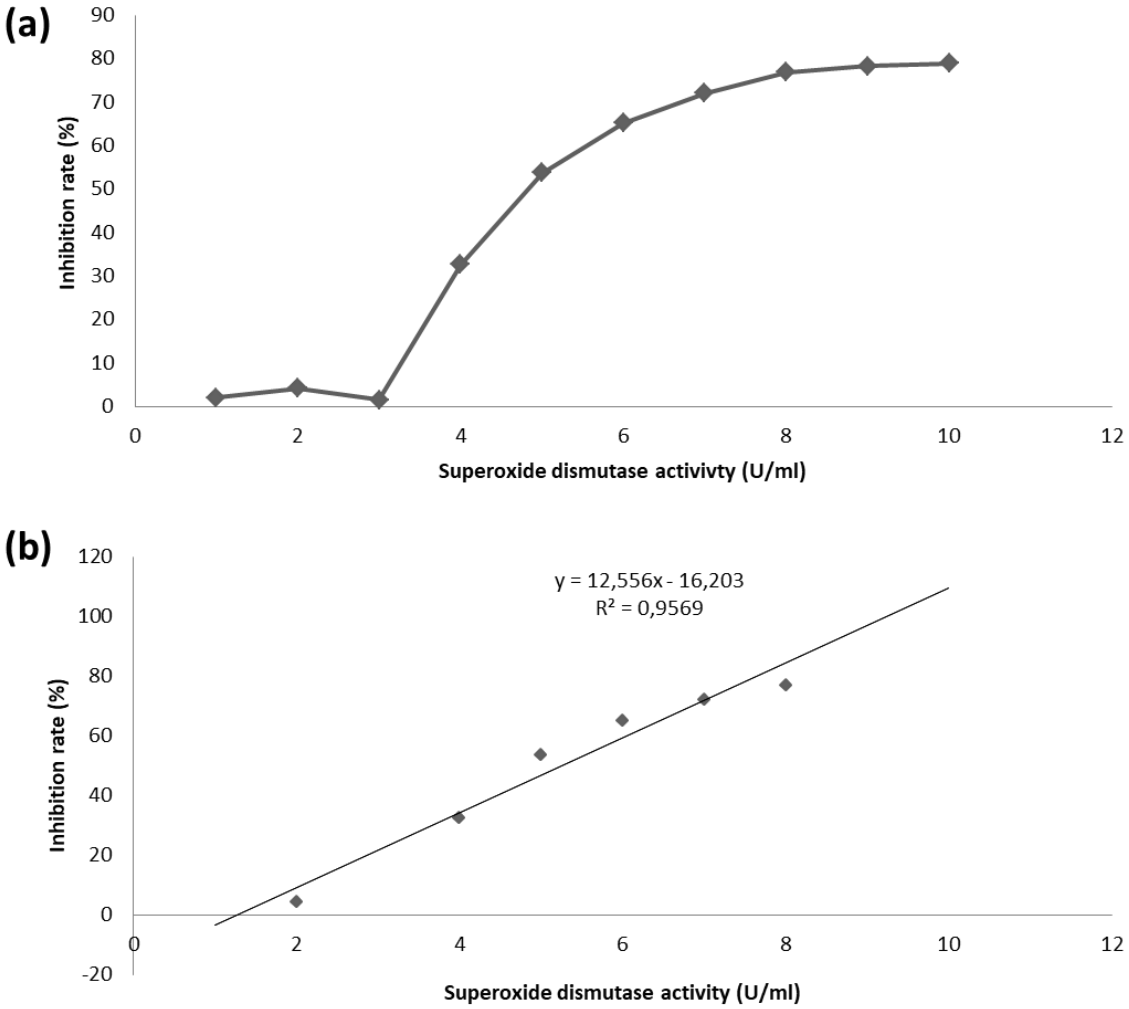

Figure S 4. (a) Standard curve and (b) standard linear curve representing inhibition rate of water soluble tetrazolium salt (WST-1) reduction by superoxide dismutase (SOD) at a detection wavelength of $OD_{450,}$ as described by Peskin and Winterbourne, (2017). SOD activity in plants were calculated using the linear equation where x = protein concentration and y = absorbance value.

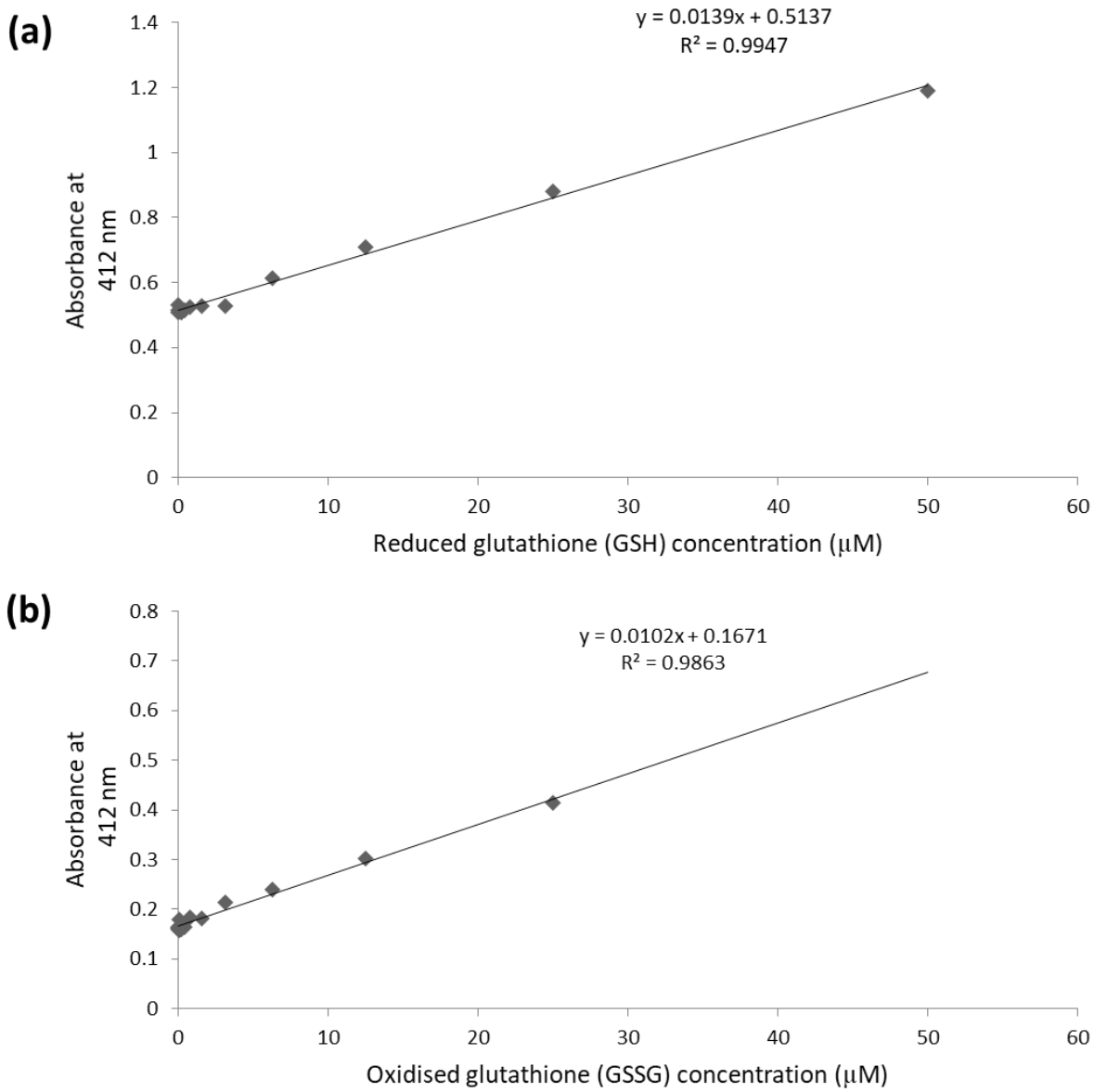

Figure S 5. Standard linear curve representing **(a)** reduced glutathione (GSH) and **(b)** oxidized glutathione (GSSH) concentration prepared as described from the method of Sahoo *et al*. (2017). Glutathione concentration in homogenate were calculated using the linear equation where x = protein concentration and y = absorbance value.

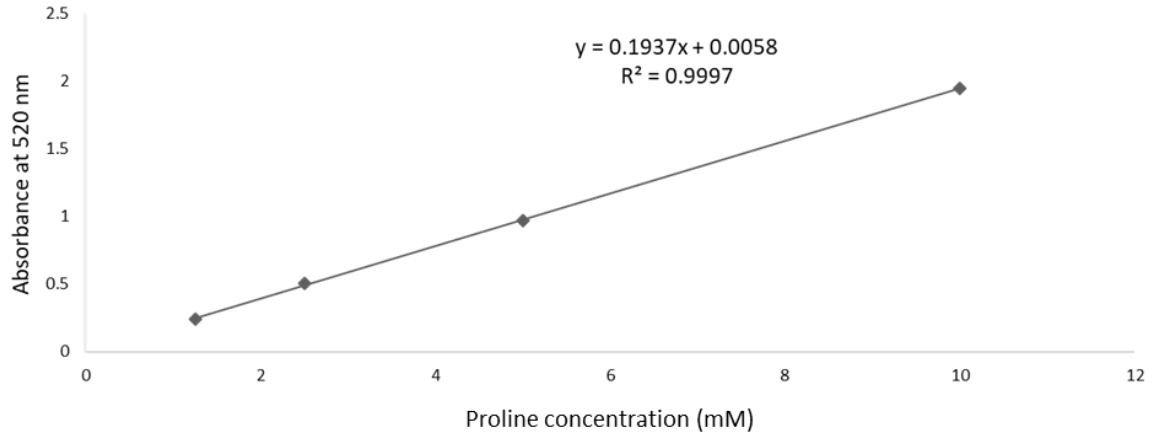

Figure S 6. Standard linear curve of proline concentration as described by the method of Bates *et al*. (1973), measured at a wavelength of OD_{520}. Protein concentration in homogenate was calculated using the linear equation where x = protein concentration and y = absorbance value.

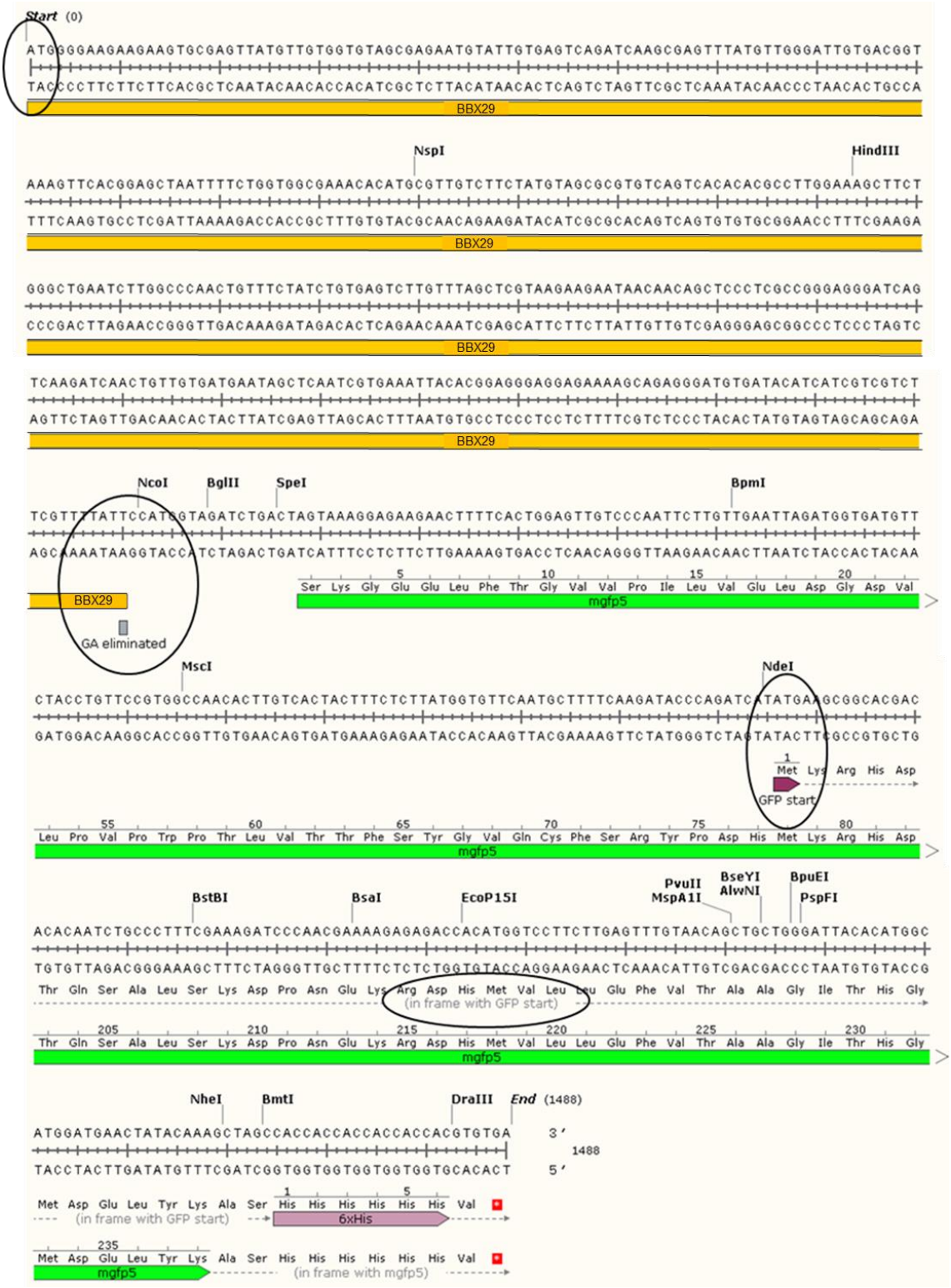

Figure S 7. DNA sequence of *AtBBX29:GFP* fusion cassette in the pCambia1302 vector. Indicated are the start codon (ATG) of the *AtBBX29* and *GFP* genes, the missing stop codon (TGA) of the *BBX29* gene and confirmation that *AtBBX29* sequence is in fame with the sequence of the *GFP* reporter gene.

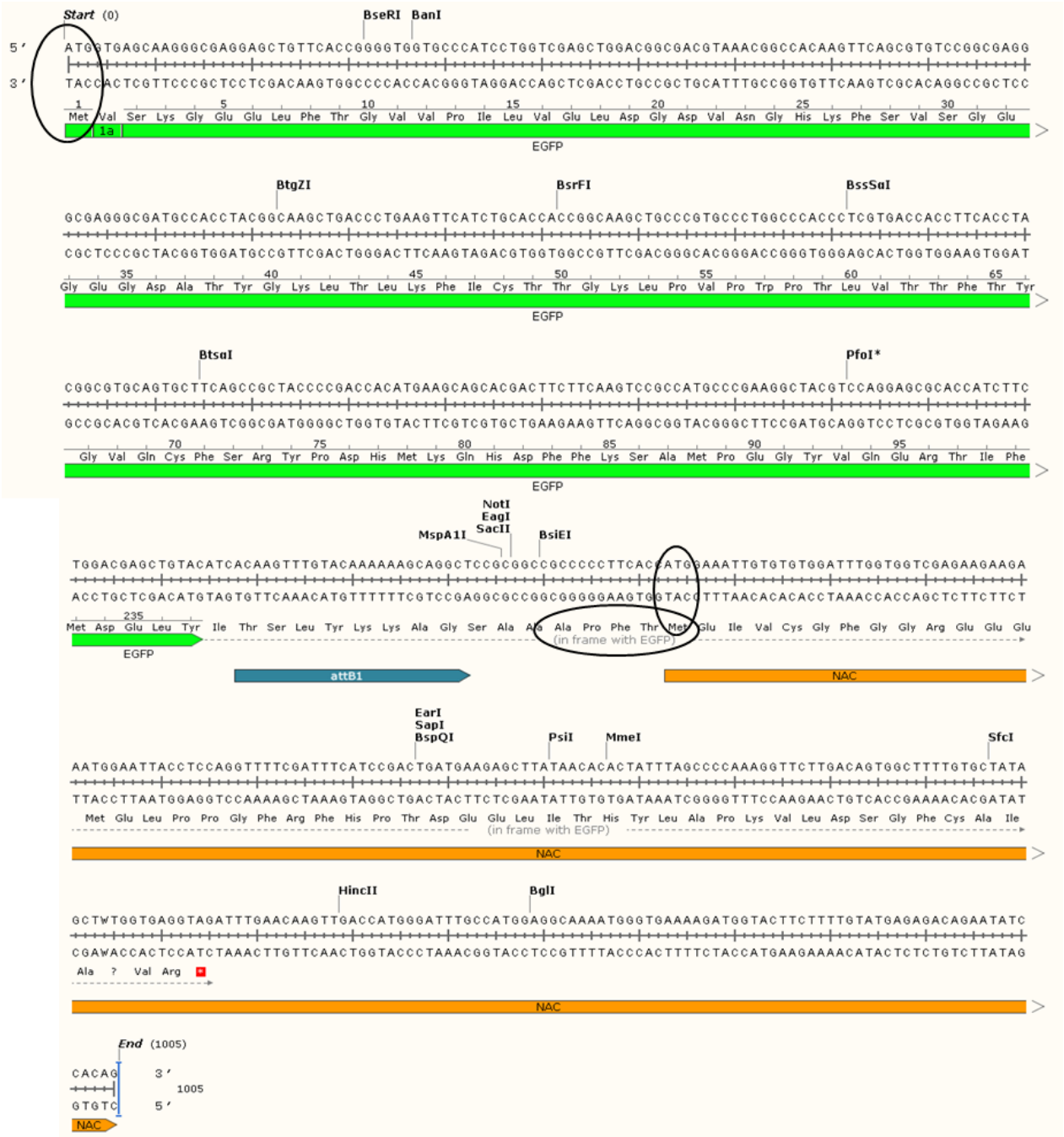

Figure S 8. DNA sequence of *GFP:SINAC2* fusion cassette in the pGWB506 vector. Indicated are the start codon (ATG) of the *SINAC2* and *GFP* genes, and confirmation that the *GFP* sequence is in fame with the sequence of *SINAC2* gene.

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